CHEMICAL SYNTHESIS, CLONING AND EXPRESSION OF A GENE ENCODING SYSTEMIN, A PROTEINASE INHIBITOR-INDUCING FACTOR.

by

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Summary

Wound-inducible proteinase inhibitors in plants elicit a defence mechanism by inactivating the proteinases of insects. This triggers a feedback mechanism causing overproduction of digestive enzymes together with a decrease in appetite, leading to starvation.

Systemin, a polypeptide proteinase inhibitor-inducing factor, when applied to cut stems of young tomato plants induces the accumulation of inhibitors in a manner similar to the normal wounding response.

We designed and synthesised the minus strand oligonucleotide template complementary to the systemin DNA sequence using Escherichia coli codon preferences. The double stranded fragment encoding the 18 amino acid residue systemin was cloned into pUC18 for amplification and subcloning into pMAL-pk for expression as a maltose binding-fusion protein. The recombinant systemin was released by enterokinase and isolated by HPLC. After further purification, the physical characteristics including amino acid composition, peptide sequence and molecular weight of r-systemin were determined. When the recombinant peptide was applied to young tomato plants, it induced the accumulation of proteinase inhibitor I messenger RNA.

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Abbreviations

A.A. (aa)	Amino acid
BSA	Bovine serum albumin
CD	Circular dichroism
CIP	Calf intestinal alkaline phosphatase
Da	Dalton
DEPC	Diethylpyrocarbonate
DIG	Digoxigenin
DMSO	dimethyl sulfoxide
DMT	Dimethoxytrityl
dNTP	Deoxyribonucleoside triphosphate
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
GCG	Genetics computer group
HPLC	High-performance liquid chromatography
HPRG	Hydroxyproline-rich glycoproteins
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IPTG	Isopropylthiogalactoside
JA	Jasmonic acid
LB	Luria broth
MALDI	Matrix assisted laser desorption/ionization
MBP	Maltose binding protein
MHz	Megahertz
NMR	Nuclear magnetic resonance
OD	Optical density
OPA	Orthophthaldehyde
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PI	Proteinase inhibitor
PIIF	Proteinase inhibitor inducing factors
POD	Peroxidase

PR	Pathogenesis-related
PTH	Phenylthiohydantoin
PVDF	Polyvinylidene difluoride
PVP	Polyvinyl pyrollidone
R.E.	Restriction enzyme
R.T.	Retention time
R.Y.	Repetitive yield
SA	Salicylic acid
SAR	Systemic acquired resistance
SBP	Systemin binding protein
SD	Standard deviation
SDS	Sodium dodecylsulphate
SE	Standard error
TEMED	N, N, N', N'-tetramethylethylenediamine
TFA	Trifluoroacetic acid
UV	Ultraviolet light

Codes for amino acids

A	Ala	alanine	**M**	Met	methionine
C	Cys	cysteine	**N**	Asn	asparagine
D	Asp	aspartic acid	**P**	Pro	proline
E	Glu	glutamic acid	**Q**	Gln	glutamine
F	Phe	phenylalanine	**R**	Arg	arginine
G	Gly	glycine	**S**	Ser	serine
H	His	histidine	**T**	Thr	threonine
I	Ile	isoleucine	**V**	Val	valine
K	Lys	lysine	**W**	Trp	tryptophan
L	Leu	leucine	**Y**	Tyr	tyrosine

Chapter 1 Introduction

1.0 Contents

1.1 Preface

The battle between crop farmers and the insects and pathogens that attack crops will never end. Traditionally, farmers have controlled crop pests and pathogens by spraying with chemical pesticides. Today, however, half of the world's insect pests are resistant to at least one major group of pesticides [10]. In future, chemical control will not be the only weapon used in this battle. One of the new strategies which scientists are currently exploring is based on transgenic plants. The plant immunity can be boosted by the transformation of either a family of genes or a single gene which encodes a natural toxin [75] or other defensive factor(s). It is therefore important to not only understand how plant defensive mechanisms operate but also to learn how to regulate and control these defensive genes.

It is known that some plants have physical or biological barriers which prevent the invasion of organisms. In addition, some crop plants also have complex chemical systems for defending themselves against their two main enemies - pathogens and insect pests. This self-defence system is somewhat similar to an animal's immune system. Many such self-defence responses have been observed in plants. One of them is called "systemic acquired resistance" or SAR response [62].

Scientists had observed the SAR response in many plant species before 1933 [15]. The SAR spectrum of resistance includes a wide variety of fungal, bacterial, and viral pathogens. SAR to pathogens usually develops after the appearance of a necrotic lesion around the inoculation site. The localised cell suicide is called the hypersensitive response (HR) which traps pathogens in and around lesions, it makes the whole plant more resistant to a wide range of disease-causing microorganisms [44]. Pathogen-induced HR and SAR are associated with the local and systemic appearance of at least five families of proteins as "pathogenesis-related" (PR) protein [41]. In 1979, White [80] demonstrated that salicylic acid (SA) and certain benzoic acid (BA) derivatives could induce both resistance and accumulation of PR proteins. SA was considered as a possible endogenous signal molecule [76].

A different system of systemic immunity triggers the accumulation of proteinase inhibitors throughout the plant in response to wounding. The proteinase inhibitors inhibit either the digestive proteinases of grazing herbivores or chewing insects by binding tightly to their target proteinases, thus blocking their activity [8, 37, 64]. The proteinase inhibitors are known to be activated systematically in plants by inducing factors such as systemin, methyl jasmonate and electrical signals but not by SA [21, 48, 65, 83].

Systemin, a plant polypeptide, was one of the inducing factors for proteinase inhibitors [55]. A brief history of its discovery and functioning will be outlined in the next section.

1.2 Proteinase inhibitors and plant defence systems

Inhibitor I from potato was first discovered and crystallised by Ryan in 1962 [66]. Later, inhibitor II from potato tubers was isolated and characterised. Initially it was thought that the synthesis of the two inhibitors was related to tuber sprouting and development but later evidence suggested that synthesis of the two inhibitors occurred predominantly in leaves of both potato and tomato. This accumulation of PIs did not relate to any developmental process, but could be induced in plants by insect damage. In 1972, Ryan found that any

severe mechanical injury of the leaves induces the synthesis and accumulation of the two proteinase inhibitor proteins not only locally but also systemically [31, 64]. These inhibitors were found to be potent inactivators of proteolytic enzymes. The presence of the inhibitors in the diets of animals can result in severe protein malnutrition and eventually in starvation [63].

Inhibitor I proteins have a molecular mass of about 8,000 Da [29], whereas inhibitor II proteins have a molecular mass of about 12,000 Da [3, 30, 71]. Both groups are serine proteinases inhibitors. Inhibitor I proteins are potent inhibitors of chymotrypsin but only weakly inhibit trypsin at its single reactive site. Inhibitor II proteins are "double-headed" inhibitors. The two reactive sites are specific for trypsin and chymotrypsin respectively [64]. In plants, both inhibitors are synthesised as precursors and undergo post-translational modification to form the mature proteins [36].

Several laboratories tried to enhance plant resistance to insect pests through gene transfer technology [4, 34]. The proteinase inhibitor I and II genes were first introduced into tobacco plants by Johnson [36] in 1989. The growth of *Manduca sexta* (tobacco hornworm) larvae was retarded when they were fed on transgenic plants which transcribed potato or tomato inhibitor II genes. However, when larvae were fed on transgenic tobacco which expressed a tomato inhibitor I gene, no reduction of growth rate was observed. Johnson concluded that the reduction of larval growth rate was due to the inhibitory activity against trypsin, not chymotrypsin [36].

In 1993, a member of the potato proteinase inhibitor II gene family that encodes an iso-inhibitor, with predominately chymotrypsin inhibitory activity rather than trypsin, was introduced into tobacco. The results from feeding trials demonstrated the usefulness of chymotrypsin inhibitors as an effective defensive agent against *Chrysodeixis eriosoma* (green looper), although they did not inhibit the growth of two closely related plusiine noctuids, *Spodoptera litura* (F.) and *Thysanoplusia orichalcea* [49].

More recently, the leaves of the transgenic tobacco plants expending potato PI-II protein were fed to *Spodoptera exigua* larvae, but the growth of the larvae was not affected. Jongasma [77] demonstrated that *S. exigua* larvae adapted to exposure to PIs by induction of new proteolytic activity which proved insensitive to PI-II. In commenting on his results, Jongasma suggested that in order to improve the resistance of transgenic plants, researchers should either look at, or design inhibitors directed against PI-insensitive proteinases complementary to the endogenous PIs in plants [77].

1.3 The induction of proteinase inhibitors

Numerous studies focused on identifying the factors which induce the synthesis of these proteinase inhibitors. Results indicated that the oligouronide fragments of plant cell walls [6, 7, 43], the plant hormone abscisic acid [57, 58], methyl jasmonate and jasmoic acid [27, 28], systemin [55], chitosan fragments from fungal cell walls [64], and electrical potentials [32, 43, 81] could be regarded as potential proteinase inhibitor inducing factors (PIIF)[64]. Subsequent research revealed the relative induction activities of these factors. These results are shown in Table 1.1 [68].

Table 1.1 Accumulation of proteinase inhibitor I in leaves* of excised tomato plants in response to various elicitors.

Elicitor	**Inhibitor I (μg/ml leaf juice)**
Water control	8
Systemin (10^{-8} M)	108
Oligogalacturonides (0.5 mg/ml)	114
Chitosan (0.1 mg/ml)	93
Jasmonic Acid (5×10^{-5} M)	119
Abscisic Acid (10^{-4} M, 1 hour)	11
Abscisic Acid (10^{-4} M, 24 hours)	9

* Young tomato plants were supplied with solutions of elicitors through the cut stem surface for 1 hour or 24 hours. The proteinase inhibitor I content was assayed by radial immunodiffusion. Adapted from Schaller [68].

A model (Fig. 1.1) presents a proposed pathway for the induction of PIs [22, 64]. The activation of defensive genes is triggered by any of the following factors: wounding; addition of systemin; oligouronides (pathogen attacks); or chitosan (fungal infection) via a lipid-derived pathway (the octadecanoid pathway). Doares [22] proposed that when all three molecules interact with their complementary plant cells membrane receptor, they start a mechanism that rapidly increases the intracellular levels of jasmonic acid (JA). JA is synthesised from linolenic acid, a major fatty acid constituent of most plant membrane lipids. The synthesis of JA leads on the transcriptional activation of the defensive genes.

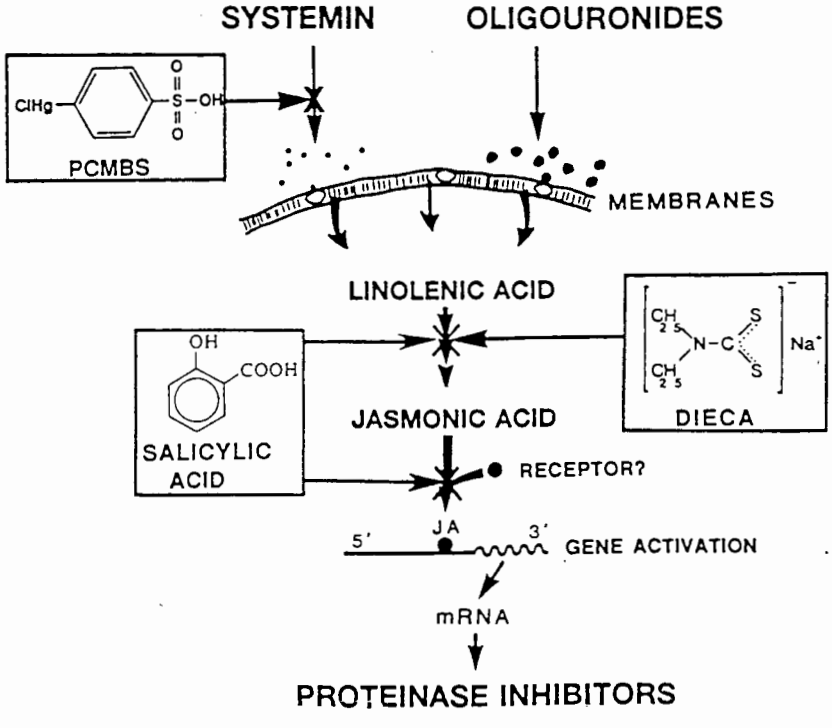


Fig. 1.1 A proposed model for the induction of PIs [22].

Doares [22] applied two inhibitors (salicylic acid and diethyldithiocarbamic acid) of the octadecanoid pathway together with systemin or oligouronides to tomato plants. The accumulation at both protein and mRNA level of PIs was reduced. When the inducing signals were supplied to the plants without the pathway inhibitors, the JA level was increased. These

findings supported the hypothesis that the primary signalling molecules activate defensive genes via the octadecanoid pathway [22].

This pathway appeared to be positively modulated by the plant hormone abscisic acid. Pena-Cortes [58] suggested that abscisic acid might activate JA biosynthesis by affecting the membrane composition, either by causing the release of fatty acids from the membrane, or by inducing enzymatic activities involved in the JA biosynthetic pathway [58].

1.4 Systemin

1.4.1 Isolation of systemin polypeptide and prosystemin gene

The polypeptide systemin was purified from tomato leaf extract by Pearce [55]. The leaf extract was separated into different fractions by using high-performance liquid chromatography (HPLC). The induction activity of each fraction was tested in young tomato plants. The fraction which exhibited the highest inducing activity was further separated using different HPLC procedures. The individual peaks were tested and analysed. A single 18-amino acid polypeptide was found to be the most effective activator. It was finally isolated and sequenced [55]:

$$^{+}H3N\text{-AVQSK}^{5}\text{PPSKR}^{10}\text{DPPKM}^{15}\text{QTD-}COO^{-}$$

The molecular mass of systemin is 2010 Da. Out of the eighteen amino acids, eleven are hydrophilic. The systemin mRNA was identified by screening a tomato cDNA library with an oligonucleotide whose sequence was derived from a partial systemin peptide sequence. The mature prosystemin mRNA is 951 bases long and possesses an open reading frame of 600 bases which encodes a 200-amino acid long prosystemin polypeptide. The initial prosystemin mRNA is encoded by a single gene (4526 bp) that consists of 11 exons and 10 introns. Prosystemin cDNA was used as a probe to hybridise the digested genomic DNA from potato,

alfafa, and *Arabidopsis thaliana* plants. A homologue of the prosystemin gene was only found in the potato [47].

McGurl [47] wounded young tomato plants and then followed the change of expression level of prosystemin mRNA in a time course experiment. The prosystemin mRNA increased to maximum expression within 3 hours after wounding, and the proteinase inhibitor I mRNA reached its highest level only 9 hours after wounding. The results proved that the systemin gene was regulated at the transcriptional level. It was further noted that prosystemin mRNA only accumulated in the aerial parts of the tomato plant [47].

1.4.2 Systemin and the induction of proteinase inhibitors

The systemin peptide is released from a 200-amino acid prosystemin polypeptide. The prosystemin molecule is hydrophilic. At the N-terminus, no hydrophobic region was found that resembled a leader sequence. The details of the proteolytic process leading to the release of the mature systemin are still unknown [47].

Synthetic systemin is as effective as the native polypeptide in inducing the accumulation of proteinase inhibitor I and II when applied to tomato plants. The level of systemin required for the induction is very low (40 fmol)[55].

Narvaez-Vasquez [51] applied the [^{14}C] labelled systemin to wounds on the surface of leaves. The whole leaf radiographic analyses revealed that [^{14}C]systemin was distributed throughout the wounded leaf within 30 minutes of its application. During the next several hours it was transported to the petiole, to the main vein, and the upper leaves. Narvaez-Vasquez compared the movement of [^{14}C]systemin and [^{14}C]sucrose applied to the wounds. Both were transported in the similar manner except that sucrose was slightly more mobile than systemin. [^{3}H]systemin distribution was observed by using a light microscope autoradiography technique. It was found that systemin was transported through phloem tissue from the

wounded leaf to other parts of plants [51]. These observations support the hypothesis that in the tomato plants, systemin is the signal which regulates systemic induction of proteinase inhibitor genes.

Pearce [56] tested the induction activity of the modified synthetic systemin, where different numbers of amino acids were deleted from either N-terminus or C-terminus. The results showed that one amino acid missing from the C-terminus of the molecule abolished induction completely. Very weak induction activity was detected for a small fragment containing only four amino acids from the C-terminus. In the substitution experiments, alanine was used to replace each amino acid individually in the molecule. The substitution of the proline (13) and threonine (17) sites totally eliminated the biological activity of systemin. These results suggested that the residues near the C-terminus of systemin were necessary for activity, probably involving phosphorylation at threonine (17). The other regions of the systemin sequence are probably important for interacting with a receptor [56].

A 50-kDa protein (SBP50), assumed to be the receptor, was identified in plasma membranes of tomato leaves. This protein binds to systemin with high specificity and resembled the furin-like prohormone convertases (a member of the family of Kex2P-like prohormone processing enzymes). A systemin derivative was labelled with biotin at residue 8 and with [^{35}S]methionine at position 15. The biotin moiety was bound by SBP50 but not the radioactive label. At least 4 amino acids from the C-terminus of systemin that included [^{35}S]methionine were missing, indicating that proteolytic cleavage had occurred [70]. Neither phosphorylation events had been associated with SBP50, nor had a functional role been associated with its proteolytic activity [22].

Orozco-Cardenas [54] transferred the prosystemin cDNA in antisense orientation into tomato plants. The growth rate of the *Manduca sexta* larvae (tobacco hornworm) fed on tomato plants which expressed the prosystemin antisense gene was about three times less than when they were fed on nontransformed control plants. The systemic induction of proteinase

inhibitor I and II in these transgenic tomato plants was very low. The results of this study suggested that prosystemin might be regulating the synthesis of other wound-inducible plant defence genes besides the PIs. The proteolysis of prosystemin might release biologically active polypeptides, other than systemin, that either activate defensive responses or other responses related to wounding [54].

Many questions about systemin activity are still lacking answers. The regulation of the prosystemin gene and the post-translational processing pathway of prosystemin in the tomato plants need to be determined. A better understanding of the role of systemin polypeptide receptors in the plant cell membrane and its downstream responses are important for the complete sequence of the proposed induction pathway. It is not yet known whether similar plant peptide hormones exist in other plants, let alone what functions they perform.

1.5 Aims of the project

In 1991, systemin, the first plant peptide hormone, was discovered and described. Its proposed biological function and its reported amino acid sequence attracted the author's interest. It was decided to use molecular biology techniques to synthesise recombinant systemin rather than manufacturing it chemically. If a pure peptide could be produced, isolated, and purified successfully, the recombinant systemin could be tested in tomato plants in order to determine its PIs inducing activity *in vivo*. Similar techniques could then be applied to express other small peptides which are difficult to synthesise or isolate. In the longer term, it was believed that it was necessary to also understand whether systemin is a signal peptide which binds to: either a membrane receptor, or the promoter region of proteinase inhibitor genes in the plants. The outline of the project is illustrated in Fig. 1.2.

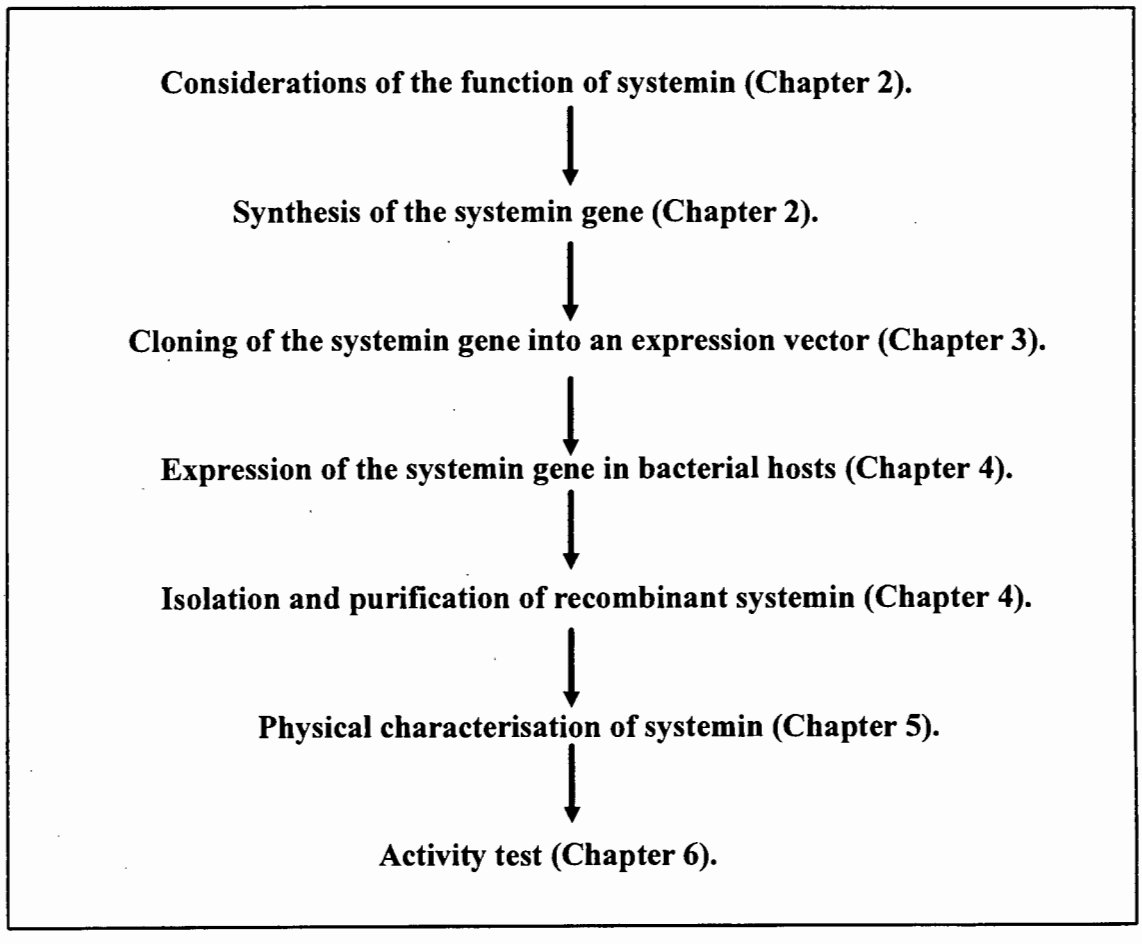


Fig. 1.2 Flow diagram of the project plans.

Chapter 2 Molecular modelling and DNA synthesis

2.0 Contents

2.1 Introduction

In order to understand and predict the biological function of systemin, we have searched protein data bases for known plant proteins which have similar peptide sequences. The actual secondary structure of systemin has been studied by using nuclear magnetic resonance (NMR) and circular dichroism (CD)[61, 73]. Here, we present a three dimensional model of systemin which is created on the computer, using energy minimisation algorithms.

Different from earlier attempts to synthesise the peptide via the peptide synthesiser, we planned to synthesise the systemin gene and clone it into a bacterial expression system to produce the systemin peptide. The procedures on which the design and synthesis of the systemin gene are based will be described in this chapter.

2.2 The primary structure of systemin

The 18 amino acid long systemin peptide sequence (Fig. 2.1) contains one "LysProPro" and one "ProProLys" motif. The most frequent amino acids are Pro (22.2%), Lys (16.7%), Ser

(11.1%), Gln (11.1%), and Asp (11.1%). 61.6% of amino acids in systemin are hydrophilic including four basic and two acid amino acids. Its estimated isoelectric point is 10.49.

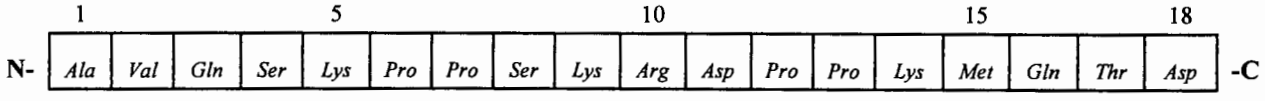


Fig. 2.1 The peptide sequence of systemin.

The GenBank™/EMBL data base was screened for known proteins with similar peptide sequences. The results showed that the "LysProPro" and "ProProLys" motifs (positions 5-7 and 12-14 of the systemin peptide) appeared in a few other plant cell wall proteins as well. These proteins included nodulin proteins, extensin and some not well classified (hydroxy)proline-rich proteins.

ENOD2 nodulin is a proline-rich protein which contributes to the structure of cell walls in pea and soybean root nodules. It is possible that ENOD2 plays a role in the development of the inner cortex of root nodules because the ENOD2 gene is specifically expressed during the formation of the inner cortex tissue. The inner cortex may function as a barrier to prevent oxygen diffusion into the nodules where the central tissue and *Rhizobium* containing cells are located. The amino acid composition of pea ENOD2 is rich in Pro, Lys, His, Tyr, Glu and poor in Ser. This polypeptide (112 amino acids) contains six "LysProPro" and "ProProLys" repeats [74].

Extensins are hydroxyproline-rich glycoproteins (HPRG) which are present in a wide variety of plants. Extensins have been isolated from carrot root, potato tuber, tobacco callus, tomato cell culture, and soybean seed coats. The carrot extensin has twelve "LysProPro" repeats in its 211 amino acids polypeptide sequence. It is highly modified and the whole molecule consists of one-third protein and two-thirds carbohydrate (w/w). The carrot extensin contains a high percentage of Hyp (hydroxyproline), Pro, Ser, Lys, His, Tyr, and Val amino acid. Its

high isoelectric point is due to the abundant Lys residues and the low frequency of Asp and Glu. Extensins have been proposed to be the major protein in the primary cell wall and to play an important role in the architecture of plant cell walls [13].

Few other proline-rich proteins have the "ProProLys" motif. For example, mRNA of a carrot proline-rich 33-kDa protein was found accumulating in wounded carrot root tissue [14]. The translated 33-kDa protein sequence has fourteen "LysProPro" repeating units. Another proline-rich glycoprotein isolated from soybean is called 1A10. The 1A10 mRNA accumulates in the cells of axis tissue of germinating soybean seed. This protein contains 23 "LysProPro" repeats in 120 amino acids [2]. An SbPRP1 protein encoding gene in soybean cell suspension culture has been reported. The predicted peptide sequence is similar to 1A10 protein. This proline-rich protein (356 amino acid) contains 43 "LysProPro" repeats [35]. These three proteins have a similar amino acid composition. They are all high in Hyp, Pro, Lys, Tyr, and Val, and low in Ser [13].

The carrot 33-kDa protein, carrot extensin, and soybean SbPRP1 protein have hydrophobic N-terminal regions which can serve as putative signal sequences. It is possible that they are secreted as extracellular cell wall proteins [35]. ENOD2 nodulin protein, 1A10, and carrot 33-kDa protein have been classified as members of a new class of cell wall proteins distinct from extensins because of the low Ser content [74].

The characteristics of systemin and other proline-rich proteins are summarised in Table 2.1. It indicates that, like the aforementioned proteins, the systemin peptide molecule has a high Pro and Lys content (Fig. 2.2) and is positively charged. However, there are differences: prosystemin does not have a significant hydrophobic N-terminal region and the mature systemin molecule is not phosphorylated or glycosylated.

Table 2.1 The characteristics of the amino acid composition of proline-rich plant polypeptides which contain ProProLys motif.

	Systemin	**Extensin**	**Nodulin**	**1A10**	**33-kDa**	**SbPRP1**
Sources	Tomato	Carrot, Potato, Tobacco, Tomato, Soybean	Pea and soybean	Soybean	Carrot	Soybean
High Hyp	–	+	**?**	+	+	+
High Lys	+	+	+	+	+	+
High His	–	+	+	–	+	–
High Tyr	–	+	+	+	+	+
High Glu	–	–	+		+	
High Ser	+	+	–	–	–	–
Glycosylation	–	**heavily**	**unknown**	+	**unknown**	**possible**
Charge	**positive**	**positive**	**positive**	**positive**	**positive**	**positive**
Solubility	+	–		+	+	+
Leading Sequence	?	+	**putative**		+	+

1 AVQS**KPP**SKRD**PPK**MQTD 18	systemin
173 PPHE**KPP**PEHQPPHE**KPP**EHQPP 195	nodulin 75-kDa
55 PPVH**KPP**SEY**KPP**VEATNSVTED 77	carrot 33-kDa
20 PRGLPG**PP**GAPGPNGFNG**PP**GEP 42	collagen

Fig. 2.2 The partial sequences of the proline-rich proteins.

2.3 The secondary structure of systemin

Two approaches were used to analyse the secondary structure of systemin. Russell [61] made the complete homonuclear proton MNR assignments and obtained the distance geometry for a synthetic systemin and its derivatives using a 500 or 600 MHz instrument. The results did not

provide any evidence for the existence of either a persistent common secondary (helix, sheet), or of tertiary structural elements in systemin, in near-neutral aqueous solution at room temperature [61].

Toumadje [73] predicted the existence of a left-handed, 3_1, poly (L-proline) II (PPII) secondary structure in the systemin peptide because of two pairs of prolines in the central region. Proline in the peptide sequence cannot be incorporated into a normal α helix structure due to the constraints imposed on the backbone by its five-membered ring. It forms a unique helical secondary structure polyproline helix II, PPII. PPII has 3 residues in one turn (3_1) and it is much more extended than α helix [12]. Toumadje used circular dichroism (CD) to scan the synthetic systemin. The CD results showed that systemin exhibited the characteristics of PPII and collagen (the PPII 3_1 structure of collagen is the best studied), but it was unlike a truly unordered peptide.

A molecular model for systemin was built by using a modelling programme, BIOGRAF. The model we created was based on energy minimisation (Fig. 2.3). However, the systemin molecule can be fairly flexible in aqueous solution at least outside the helical central region [73]. It is possible that systemin binds either to other protein(s) or to other factor(s) and forms a stable secondary structure (Dr Mark Conkling, personal communication).

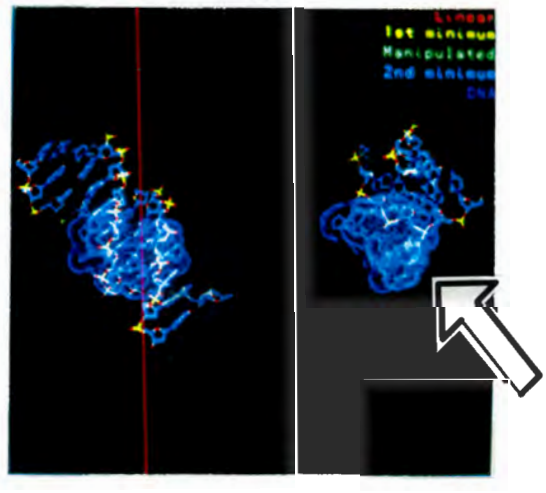


Fig. 2.3 3D model of systemin. The DNA double helix model is shown for comparison of size.

2.4 Design of oligonucleotides and PCR

The mature systemin peptide is released from a 200 amino acid systemin precursor protein in tomato plants. We could not clone its cDNA to express systemin in bacterial hosts. The alternative was to synthesise the coding region of systemin chemically. A single-stranded oligonucleotide coding for systemin peptide was designed, synthesised, and amplified from a single-stranded oligonucleotide into a double-stranded form for cloning, using a PCR method.

The PCR method of Michaels [50] is suitable for the amplification of long synthetic oligonucleotides. Traditionally during the chemical synthesis the yield of the full-length oligonucleotide (125-150 bases) is lower than 1% due to the low coupling efficiency [24]. It is necessary to purify the full-length product for gene cloning. This PCR method, however, uses two overhanging primers which anneal preferably to the full-length template at high annealing temperature. This design leads to preferential amplification of the minute amount of full-length product present in mixtures of aborted oligonucleotides [50].

2.4.1 Design and synthesis of oligonucleotides

A fifty-seven base long oligonucleotide encoding the systemin peptide with an additional stop codon at the 3' end was designed using *E. coli* codon preferences [78], as the difference between tomato and *E. coli* codon usage frequency might affect (Fig. 2.9), however, the translation rate and accuracy [72]. Special restriction sites were added to the 5' end of the two overhang primers for cloning. An additional three bases at the 5' end of the primers offered some space for restriction enzymes to recognise the sites and digest successfully. General rules were followed for primer design (Chapter 8), for instance, the length of primer was between 20 to 30 bases, the GC content was about 50%, no significant secondary structure appeared to exist at the 3' ends, and no obvious complementarity at the 3' ends of the two primers which could lead to "primer-dimers" [26].

The annealing temperature in the PCR was expected to change after the second cycle because the template extended from 57 bases to 78 bases after the first PCR extension (Fig. 2.5-2.8). Not only the overlap region but the whole primer would anneal to the template. The *Tm* of template/primer complex was calculated using the following two formulas:

1. Tm (°C) = 4 × (number of G+C) + 2 × (number of A+T)

This formula was originally calculated for helices dissolved in a 1 M salt concentration used in oligonucleotide hybridisation assays [9, 20]. However, the result was inaccurate for complexes longer than 20 nucleotides.

2. Tp (°C) = 22 + 1.46 (ln)

where *Tp* = optimised annealing temperature ± 2-5°C, *ln* = effective length of primer = 2 × (number of G+C) + (number of A+T). This formula was suitable to calculate *Tm* for oligonucleotide helices of 20-35 nucleotides in length [82]. The above formulas were used in our primer design (Table 2.2).

Table 2.2 The annealing temperatures of the primers in different PCR cycles.

	GC%	Tm (°C) = 4 (G+C) +2 (A+T)	Tp (°C) = 22+1.46 (ln)
Primer 1			
29 bases*	**65.5**	**96**	**92.1**
12 bases**	**58.3**	**38**	**50**
Primer 2			
22 bases*	**59**	**70**	**73.1**
12 bases**	**50**	**36**	**48.3**

* Full length of primers. ** The overlap region

The oligonucleotide sequences of template and primers were examined for secondary structure formation by using the "Fold" programme in GCG before synthesis (Fig. 2.4). Any potential secondary structure in this sequence which might interfere with the PCR were avoided by using alternative codons for the most favourable ones. Most of the secondary structures would be broken down at high denaturing temperature.

All three oligonucleotides were synthesised on an AutoGen 6500 DNA synthesiser. The 5'-dimethoxytrityl (DMT) protecting groups at the 5' end of oligonucleotides were removed. The purity of oligonucleotides was examined on a denaturing acrylamide gel.

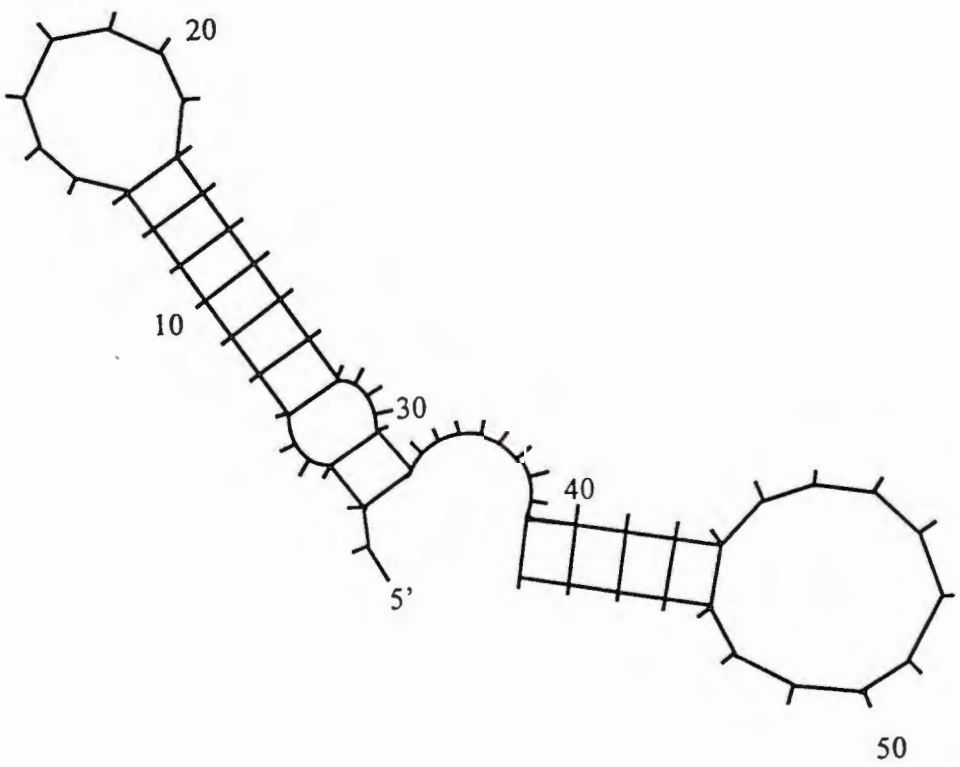


Fig. 2.4 The secondary structure of the PCR template.

Primer 1: 29 bases
5' AAGCCCGGGTGC[GCAGTGCAGAGCAAACC] 3'

Fig. 2.5

Minus strand template: 57 bases
3' CGCCACGTCTCGTTTGGCGGCTCGTTTGCACTAGGCGGCTTTTACGTCTGGCTAATT 5'

Fig. 2.6

Primer 2: 22 bases
3' [CGTCTGGCTAATT]GGGCCCGAT 5'

Fig. 2.7

[ ]: overlapping region of primer and template

Full length PCR product: 78 bp

Fig. 2.8

```
      SmaI     AvilI                                   Sau3A                          SmaI
5' AAGCCCGGGTGC{GCAGTGCAGAGCAAACCGCCGAGCAAACGTGATCCGCCGAAAATGCAGACCGATTAA}CCCGGGACT 3'
                 Ala  Val  Gln  Ser  Lys  Pro  Pro  Ser  Lys  Arg  Asp  Pro  Pro  Lys  Met  Gln  Thr  Asp  *
```

* Stop codon; { } coding region

Similarity of designed and original DNA sequences: 66.7%

Fig. 2.9

```
Systemin gene (tomato): 5' GCTGTTCAATCAAAACCTCCATCAAAGCGTGATCCTCCCAAAATGCAAACAGAC 3'
                           ||  ||  ||     |||||  ||     ||  ||||||||  ||  ||||||||  ||  ||
Designed systemin gene: 5'GCAGTGCAGAGCAAACCGCCGAGCAAACGTGATCCGCCGAAAATGCAGACCGAT 3'
```

2.4.2 PCR

Some factors which might influence the efficiency and specificity of PCR were optimised: for example, the quality and concentration of template and primers; concentration of the deoxyribo-nucleoside triphosphates mixture (dNTPs); magnesium ion concentration; use of thermostable DNA polymerases; PCR thermal profile (primer annealing, extension, denaturation temperatures, ramp times and number of cycle)[53].

Vent DNA polymerase isolated from *Thermococcus litoralis* was used instead of *Taq* DNA polymerase in this PCR. *Thermococcus litoralis* is a thermophilic archaebacterium found on ocean floors at temperatures of up to 98°C. Vent DNA polymerase is more thermostable than *Taq* DNA polymerase and it possesses a 3' to 5' exonuclease activity which improves the fidelity [53].

All reaction solutions and instruments were kept sterile. DNA, dNTPs, polymerase and buffer were stored at -20°C. The reaction vials were kept on ice during preparation. Airborne contamination was avoided by quick preparation and closing the lids whenever the vials were not used. Wearing gloves was essential in order to prevent proteinase or nuclease contamination from the hands.

2.4.3 PCR cycle profile

Generally, PCR is performed by incubating the samples at three different temperatures corresponding to the three steps in a cycle namely: denaturation, annealing, and extension. In a typical reaction, the double-stranded DNA is denatured briefly by heating the samples to 90-95°C, the primers are allowed to anneal to their complementary sequences by briefly cooling to 40-60°C, followed by heating to 70-75°C to extend the annealed primers with DNA polymerase [26].

In the first cycle of our PCR, the single-strand oligonucleotide was denatured at 92°C for 20 sec, then primer 1 was allowed to anneal to the template at 65°C for 30 sec and to extend at 72°C for 60 sec. From the second cycle onwards, both primers were allowed to anneal and to extend on the template which was synthesised in the first cycle. The cycle was repeated 35 times to produce a large quantity of the double-stranded PCR product.

2.4.4 Analysis of the PCR product

After amplification, the PCR mixture was digested with restriction endonucleases. Different enzyme digestions released different numbers and sizes of DNA fragments. Because our DNA fragments were relatively small (36 bp to 78 bp), a high resolution gel system had to be used to analyse the digestion results. The following formula was applied to determine the relationship between migration distance and molecular weight.

$$D = a - b (\log M)$$

where "D" is the distance the DNA migrated, "M" is the molecular weight, and "a" and "b" are constants which depend on electrophoresis conditions. A simpler but less precise way of estimating DNA fragments sizes is by using a standard curve (log DNA nucleotide number versus distance migrated)[11].

The sequence of the amplified DNA fragment and its restriction enzyme map is shown in Fig. 2.8. The restriction enzyme SmaI cuts twice (on the ends of PCR product) to produce three small fragments 66 bp, 6 bp and 6 bp. Another endonuclease, Sau3A cuts once and produces two fragments which are 42 bp and 36 bp long.

2.4.5 Results of PCR

12% denaturing acrylamide gels were run to analyse the restriction endonuclease fragments (Fig. 2.10). High quality acrylamide and bisacrylamide solution were used to create a matrix which generated a good resolution. Hpa II digested pBR322 plasmid was used as the molecular weight marker. The size of the marker fragments ranged from 9 bp to 622 bp. The gel was stained in a 10 μg/ml of EtBr solution for five minutes to visualise the DNA bands. The DNA sizes were estimated by using the standard curve method (Chapter 2, §4.4). The relationship between the distance travelled by the DNA in the gel (mm) and the size of the corresponding oligonucleotides is shown (Fig. 2.11).

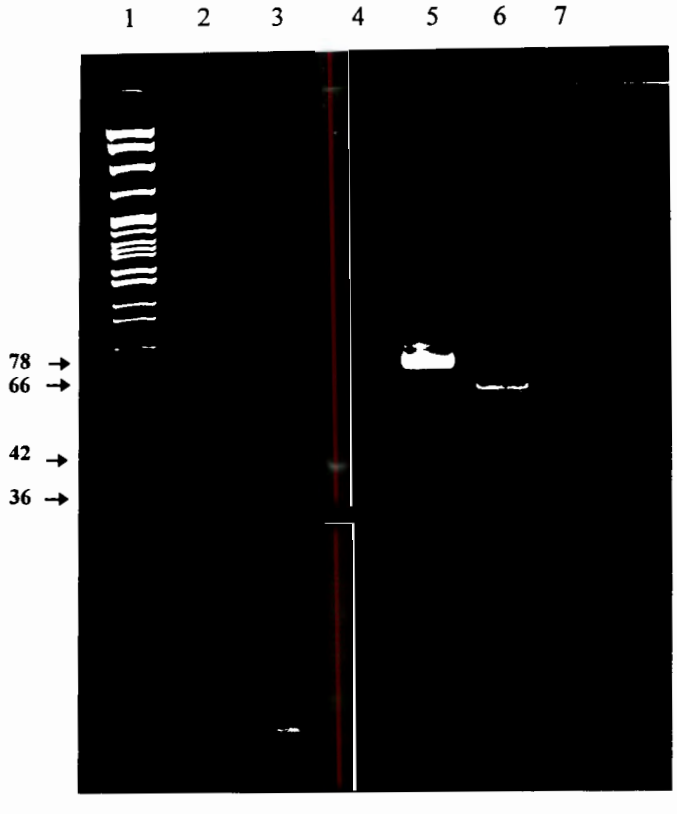


Fig. 2.10 Restriction analysis of PCR product. Lanes: 1. pBR322 cut with HpaII; 2. ss primer 1 (29 bases); 3. ss primer 2 (22 bases); 4. ss template (57 bases); 5. ds full-length PCR product (78 bp); 6. ds SmaI digested PCR product (66 bp); 7. ds Sau3A digested PCR product (36 bp and 42 bp).

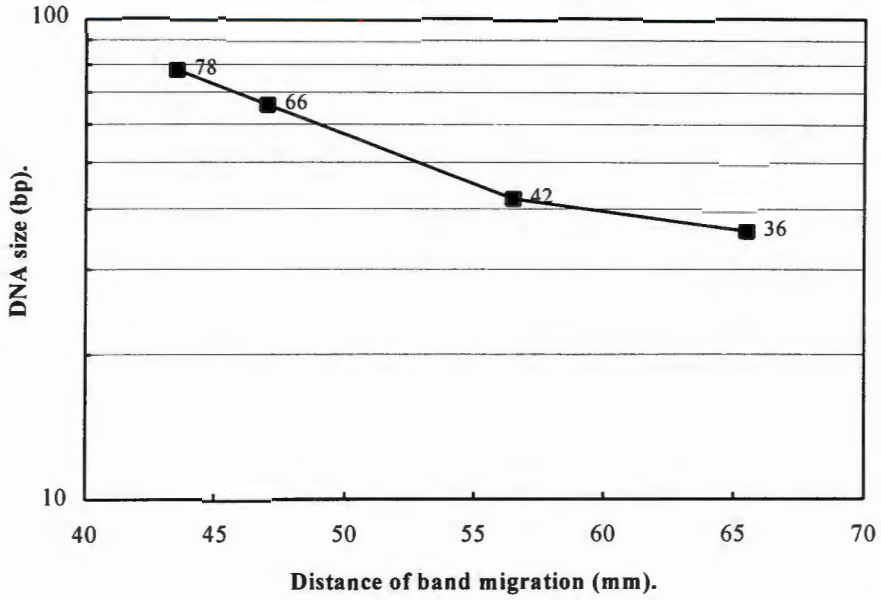


Fig. 2.11 Standard curve relating DNA sizes and migration distances on a denaturing acrylamide gel.

2.5 Discussion and summary

We screened a protein data bank for proteins that had similar peptide sequences to systemin. A few plant cell wall or proposed cell wall proteins were found, which contain either a repeated "ProProLys" or "LysProPro" motif like systemin. These proteins are all proline-rich and mostly highly modified (phosphorylated and/or glycosylated), and they are all basic with high isoelectric points. However, unlike these proteins, none of the proline in the systemin peptide sequence is modified and no N-terminal hydrophobic region is found in the prosystemin peptide sequence. More evidence is needed to determine whether systemin is a cell wall protein or not.

Although systemin did not show a persistent secondary structure in NMR, a left-handed, 3_1, poly (L-proline) II secondary structure was suggested for the central region of systemin [73]. The results of other *in vivo* tests showed the importance of the C-terminal part of systemin. When a synthetic peptide consisting of the last 4 residues of the C-terminus was applied to tomato plants, a very weak proteinase inhibitor induction response was detected. The induction activity was completely lost if one amino acid from C-terminus of the peptide was missing [56]. A 50-kDa systemin binding protein (SBP50) was isolated from the tomato plasma membrane by Schaller [70]. SBP50 cleaved the systemin peptide at the position at least 4 amino acids from the C-terminus. It seems that the amino acids at the N-terminus of the molecule are the important residues for proper binding. But the residues responsible for proteinase inhibitor induction are located in the C-terminus [70].

Oligonucleotides for PCR amplification of a synthetic systemin gene were designed and synthesised. The size of the PCR product and its restriction endonuclease fragment map were examined on the denaturing acrylamide gels before the PCR product was cloned into pUC18 plasmid.

Chapter 3 Cloning and subcloning of systemin gene

3.0 Contents

3.1 Introduction

Gene cloning is used widely in biochemical research [11]. In the first step, a fragment of double-stranded DNA, containing the gene to be cloned with suitable restriction sequences at the ends, is inserted into a circular DNA molecule called a vector, to produce a recombinant DNA molecule. The vector can then be transported into a host cell. Within the host cell the vector is multiplied, thus producing numerous identical copies not only of itself but also of the gene that it carries. When the host cell divides, the host genomic DNA and few copies of the recombinant DNA molecules are passed to the progeny and following further vector divisions, a colony, or clone of identical host cells is produced. Each cell in the colony contains one or more copies of the recombinant molecule; the inserted gene carried by the recombinant molecule is now said to be cloned.

Gene cloning follows a standard protocol.

1. Pure DNA samples are prepared. A large-scale preparation method is used to isolate the vectors from host cells (Chapter 8).

2. Complementary termini of the insert and vector are generated by restriction enzyme digestion. The DNA fragments are examined on either an agarose gel or an

acrylamide gel and compared to the molecular weight marker.

3. The vector termini are dephosphorylated by phosphatase. Calf intestinal alkaline phosphatase (CIP) is usually the enzyme of choice to remove 5'-phosphate groups from linear plasmids.

4. The vector and insert are joined together by ligase. Ligation of a segment of foreign DNA to a linearised plasmid vector involves the formation of new bonds between phosphate residues located at the 5' termini of double-stranded DNA and adjacent 3'-hydroxyl moieties. When both strands of the plasmid vector carry 5'-phosphate residues, four new phosphodiester bonds must be generated. However, when the plasmid DNA has been dephosphorylated, only two phosphodiester bonds can be formed. The resulting hybrid molecules carry two single-stranded nicks that are repaired after the hybrids have been introduced into competent bacterial cells. The complementary ends of a plasmid will not be able to ligate themselves if the 5'-phosphate groups have been removed [46].

5. The recombinant DNA molecule is transformed into a competent host cell. Many strands of *E. coli* cells with different characters can be chosen from. Competent *E. coli* cells have a higher efficiency of transformation. The salt in the competent cells buffer possibly either causes the DNA to precipitate onto the outside of the cells, or it is responsible for some kind of change in the cell wall that improves DNA binding [11].

6. The transformed cells containing recombinant plasmid are distinguished from the transformed cells that contain normal plasmids. The following methods can be used: the insertional inactivation of a selectable marker [11]; colony hybridisation probing [11]; PCR screening [79]; and restriction mapping [46].

7. The recombinant DNA is sequenced to determine the sequence of the insert [11].

3.2 Gene Cloning

The PCR synthesised double-stranded systemin gene was first cloned into a pUC18 plasmid and then subcloned into a modified pMAL-p vector for gene expression.

3.2.1 Cloning of the systemin gene into the pUC18 plasmid.

pUC18 (2.6 kb) is a small plasmid which consists of a pBR322 derived β-lactamase gene and an origin of DNA replication ligated to the α portion of the *lacZ* gene of *E. coli.* The pUC plasmids can grow to high copy numbers [18].

Before ligation, restriction enzyme Sma I was used to linearise the close-circular pUC18 plasmid and to digest the PCR product (Chapter 2). The linearised plasmids were dephosphorylated by calf intestine phosphatase. Six different ligation samples were prepared. Five of them were negative controls or positive controls for the ligation and transformation experiments. The blunt-end ligation was carried out at 22°C overnight. The competent *E. coli* cells (HB101) were then used for transformation. Finally, the transformed cells were spread on agar plates and incubated at 37°C overnight. The colony number from different ligation samples were counted the following day (Table 3.1).

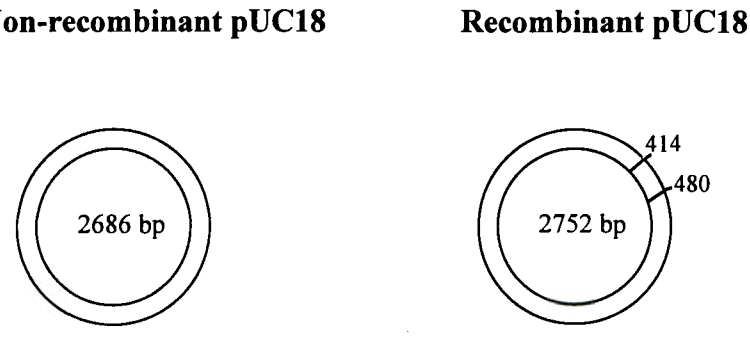
Table 3.1 The results of transformation.

Transformation samples	**Colony numbers**
No plasmid	0
Supercoiled plasmid	> 800
Linear plasmid	3
Linear plasmid with ligase	438
CIP linear plasmid with ligase	0
CIP linear plasmid and insert with ligase	13

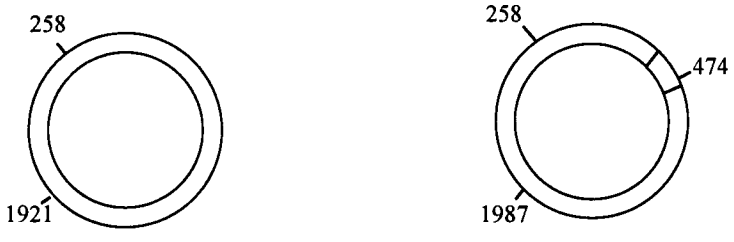
0.2 pmole of plasmid and 0.4 pmole of insert were ligated and transformed.

To perform a colony screening, the oligonucleotide DNA encoding for systemin was labelled with radioactive [^{32}P]. The plasmids were denatured and fixed onto the solid support for hybridisation. If the plasmid contained the recombinant sequence, the labelled single-stranded DNA would anneal to its complementary strand in the insert. The hybridisation was detected by using autoradiography to select for positive colonies. The plasmids were isolated from amplified *E. coli* cells of these colonies for restriction enzyme mapping (Chapter 8).

The restriction enzyme SmaI cut once in a non-recombinant pUC18 DNA, and twice when the systemin gene was inserted. AviII contained two recognition sites in a pUC18 plasmid. One more recognition site would be generated if the insert was cloned into pUC18. In a double digestion of plasmid with SmaI and AviII, the recombinants released 5 fragments of the following sizes: 6 bp, 60 bp, 156 bp, 1023 bp, and 1507 bp (Fig. 3.1), and 3 fragments produced if the plasmid was non-recombinant. The sizes of the fragments were examined on the agarose gels (Fig. 3.2).



A. SmaI digestion



B. AviII digestion

C. AviII/ SmaI digestion

Fig. 3.1 The restriction maps of pUC18 with or without insert.

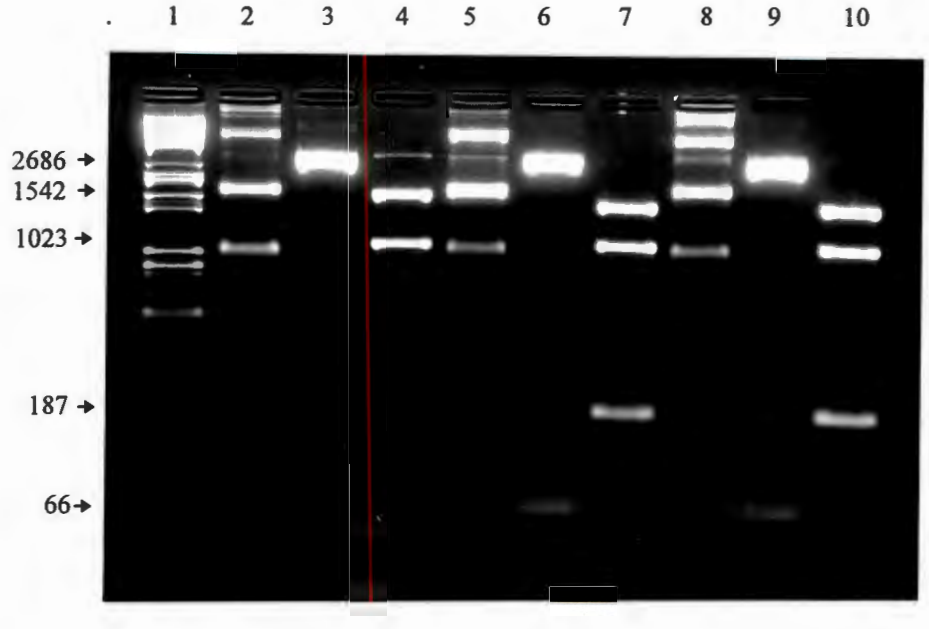


Fig. 3.2 Restriction analysis of the recombinant plasmid: DNA fragments are separated by electrophoresis through a 1.7% agarose gel. Lanes: 1. EcoRI/HindIII digested marker; 2. supercoiled pUC18; 3. linearised pUC18 cut with SmaI; 4. pUC18 cut with AviII (1023 and 1663 bp); 5, 8. supercoiled recombinant pUC18; 6, 9. recombinant pUC18 cut with SmaI into two fragments (66 and 2686 bp); 7, 10. recombinant pUC18 cut with AviII into three fragments (216, 1023, and 1513 bp).

The recombinant pUC18 plasmid was sequenced by using the TaqTrack™ sequencing method (Promega technical manual). The high reaction temperature (70-80°C) of *Taq* DNA polymerase decreased the secondary structure of DNA templates and thus allowed polymerisation through highly structured regions. The primers were designed and

synthesised. For the sequencing reaction, the radioactive labelled [^{35}S]dATP was mixed with unlabelled dNTPs, which was incorporated into the DNA chain during polymerisation. Autoradiography was performed to distinguish the DNA fragments on the sequencing gel.

Once the sequence of the insert was verified, the recombinant plasmids were digested with AviII and SmaI to release the systemin gene. The digestion mixture was loaded onto a 12% non-denaturing acrylamide gel for gel purification, and the 60 bp DNA fragment was eluted from the gel [1].

3.3.2 Modification of pMAL-p/c plasmid to pMAL-pk/ck.

pMAL vectors allow the expression and purification of a protein of interest by fusing it to the maltose binding protein (MBP) which is encoded by the *Mal*E gene of *E. coli* [23]. pMAL vectors have a strong *tac* promoter and a *mal*E translation initiation signal to give high-level expression of the cloned sequences. The vector expressing the *mal*E gene is fused to the *lacZα* gene. Sites between *mal*E and *lacZα* are available for inserting the coding sequence of interest, which interrupts the *mal*E-*lacZα* fusion [1].

pMAL-p expresses the MBP containing its original signal sequence, so the fusion protein will be exported to the periplasm of *E. coli*. This process promotes proper folding and disulfide bond formation for some proteins, and allows purification from the periplasm. The *mal*E gene of pMAL-c has a deletion of the signal sequence, leading to cytoplasmic expression of the fusion protein [1].

The pMAL vectors also carry the *Lac*I gene, which codes for the *lac* repressor. This design keeps the expression of the *tac* promoter low in the absence of isopropyl-1-thio-β-*D*-galactoside (IPTG) induction. There is a sequence coding for the recognition site of protease factor Xa which is located 5' to the polylinker insertion sites. This site allows MBP to be cleaved from the protein of interest with factor Xa after purification [Manual C].

Factor Xa cleaves after its four amino acid recognition site, IleGluGlyArg [1, 45]. The pMAL-p/c plasmid was modified by adding an enterokinase recognition site after the factor Xa site. Enterokinase is a serine protease also named enteropeptidase. *In vivo*, enterokinase activates its natural substrate trypsinogen and releases trypsin by cleavage at the C-terminal end of the pentapeptide sequence AspAspAspAspLys [40]. Two complementary single-stranded oligonucleotides were designed which encode the above pentapeptide sequence (Fig. 3.3). The annealed double-stranded DNA would be inserted into the pMAL plasmid.

```
+ strand (21 bases):      5' AGCGGGGATGACGATGACAAG 3'
- strand (25 bases):      3' TCGCCCCTACTGCTACTGTTCTTAA 5'
peptide sequence:          N-SerGlyAspAspAspAspLys-C
                                   enterokinase cleavage site
```

Fig. 3.3 The complementary single-stranded oligonucleotide sequences.

These two single-stranded DNA molecules were incubated in a buffer containing 66 mM Tris·HCl, pH 7.5, 1 mM DTT, 5 mM $MgCl_2$ at 90°C for 2 minutes, then cooled down slowly at room temperature (Jens Völker personal communication). The result of the annealing was examined on a 12% denaturing acrylamide gel (Fig. 3.4). The annealed double-stranded DNA had one blunt end and one sticky end which was complementary to an EcoRI digested end.

Fig. 3.4 12% denaturing polyacrylamide gel analysis of the annealing of two single-stranded oligonucleotides. Lanes; 1. pBR322 cut with HpaII; 2. coding strand of EKS (enterokinase cleavage site sequence); 3. non-coding strand of EKS; 4. double-stranded EKS.

pMAL-p/c plasmids were digested by StuI and EcoRI. The linearised plasmid contained one sticky end and one blunt end. The plasmid had been dephosphorylated by CIP. The plasmids and the synthetic double-stranded DNA fragment were ligated at 12°C overnight (Fig. 3.5). The ligated samples were transformed into competent *E. coli* host cells (TB1). The transformation result is shown in Table 3.2.

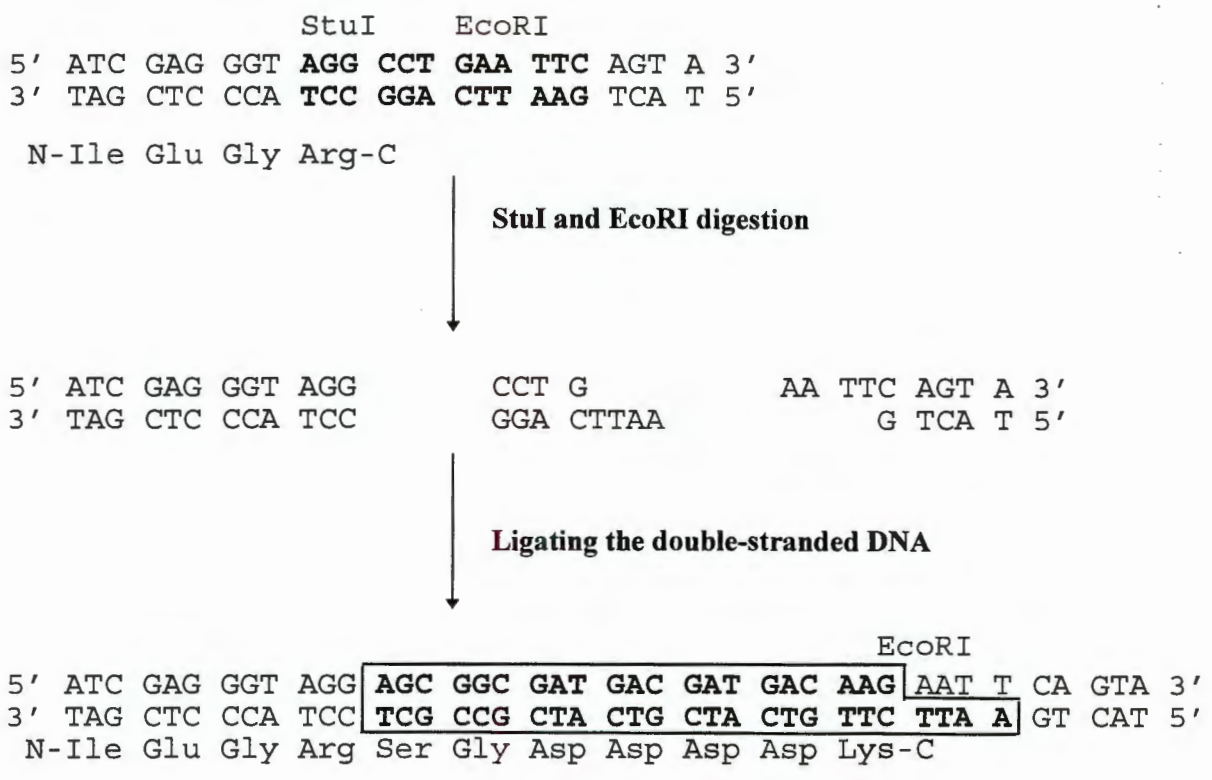


Fig. 3.5 The ligation procedures.

Table 3.2 The results of transformation.

Transformation samples	**Colony numbers (pMAL-p)**	**Colony numbers (pMAL-c)**
No plasmid	0	0
Supercoiled plasmid	335	713
Linear plasmid	0	0
CIP linear plasmid with ligase	0	0
CIP linear plasmid and insert with ligase	24	26

0.02 pmole of plasmid and 0.4 pmole of insert were ligated and transformed.

The colonies were screened by a PCR screening method [79]. In the PCR reaction, two primers located at the ends of the pMAL vector multicloning site extended and produced a DNA fragment with the length between two primers. The recombinant plasmid produced a

PCR DNA fragment 17 bp longer than non-recombinant plasmid. The difference of the DNA fragments was examined on a 3% agarose gel.

For restriction mapping, EcoRI and StuI were used to digest the plasmids. The EcoRI recognition site still existed after the ligation of the double-stranded DNA fragment, but the StuI site was missing. The pMAL-p/c plasmid could be cleaved by either EcoRI or StuI, but the recombinant plasmid could only be linearised by EcoRI.

The nucleotide sequence of the insert was determined with the same method as described in Chapter 3, §3.1. The pMAL-p and pMAL-c plasmids containing the enterokinase recognising site were named pMAL-pk and pMAL-ck respectively.

3.2.3 Subcloning the systemin gene into pMAL-pk/ck plasmid.

The 60 bp DNA fragment containing the systemin gene was eluted from a non-denaturing acrylamide gel (Chapter 3, §2.1). The pMAL-pk and ck plasmid were digested by EcoRI to generate a linearised plasmid each with two sticky ends. The sticky ends were blunt-ended by using a single strand specific exonuclease, nuclease P1 [10]. Nuclease P1 cleaves 5' phosphodiester bonds and 3' phosphodiester bonds after nucleotide sequence A, C, G, U, or T. The incubation time and the concentration ratio of linearised plasmid to nuclease P1 were optimised in order to prevent overdigestion (Fig. 3.6).

The systemin gene was ligated to blunt-ended pMAL-pk and pMAL-ck at 22°C overnight. The ligated plasmids were transformed into competent TB1 cells. The colony numbers of different ligations are shown in Table 3.3.

The transformed *E. coli* colonies were screened by using a PCR screening method. The PCR fragment of the recombinant plasmid was 56 bp longer than the plasmid without insert

(Fig. 3.7). The recombinant plasmids were sequenced to check the direction of insertion.

Ten colonies were screened. Of these, two out of four pMAL-ck colonies, and four out of six pMAL-pk colonies had a longer PCR fragment. However, it was found that only one recombinant pMAL-pk contained the correct insert after nucleotide sequencing.

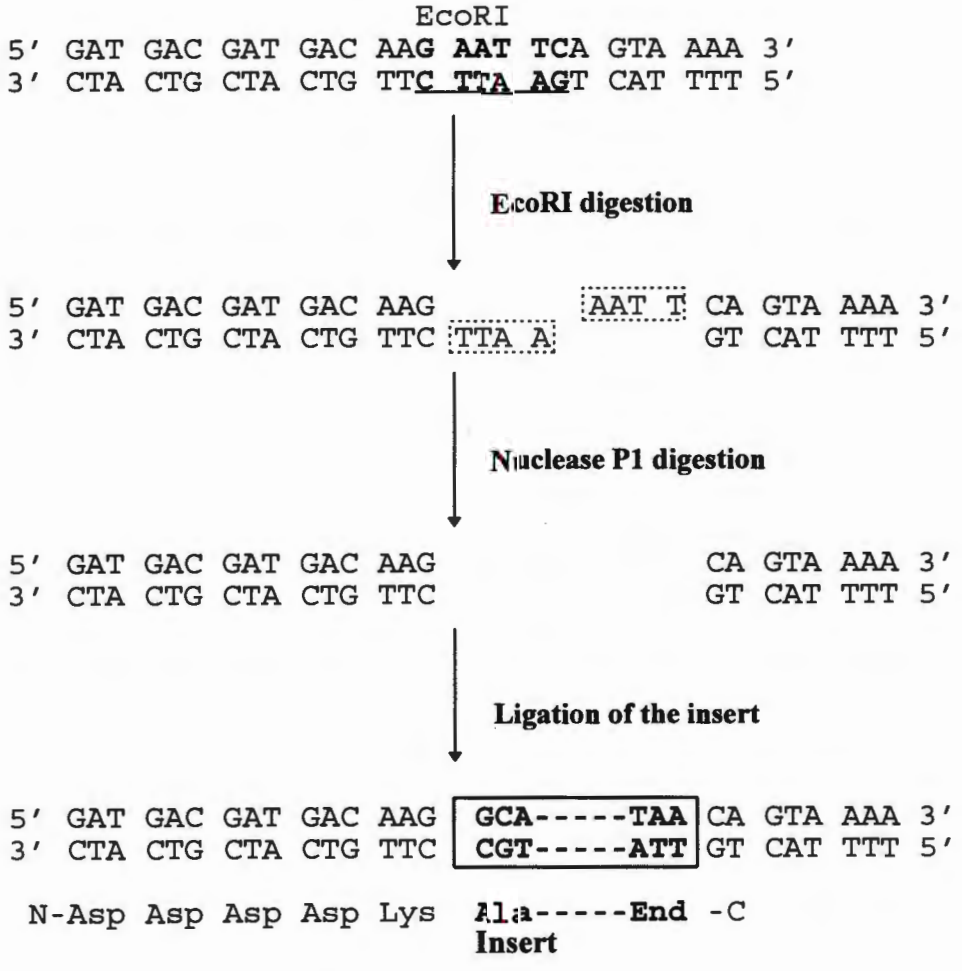


Fig. 3.6 The ligation procedures.

Table 3.3 The results of transformation.

Transformation samples	**Colony numbers of pMAL-ck ligation**	**Colony numbers of pMAL-pk ligation**
Supercoiled plasmid	1601	1720
Linearised plasmid and ligase	911	1175
CIP plasmid and insert and ligase	4	6

0.01 pmole of plasmid and 0.08 pmole of insert were ligated and transformed.

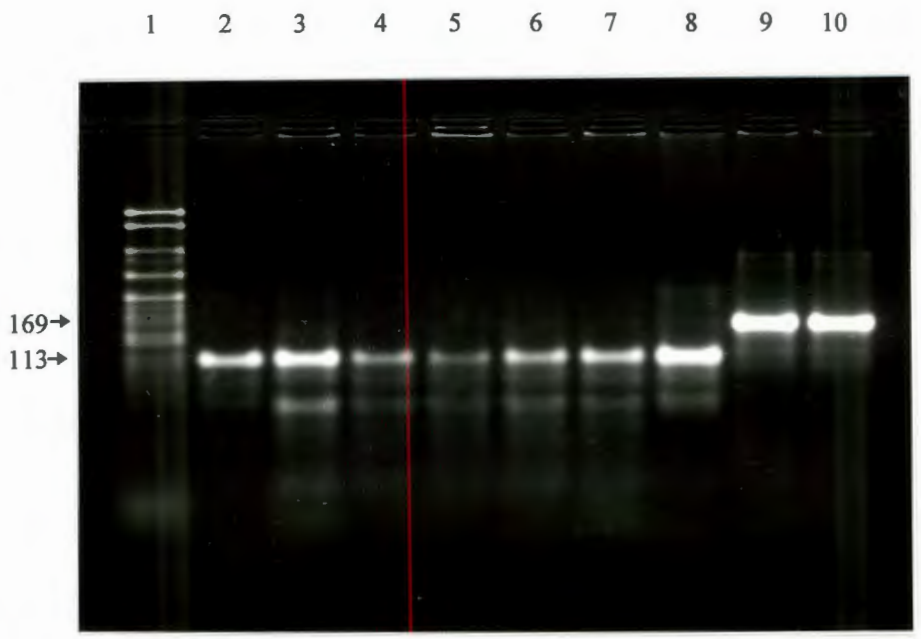


Fig. 3.7 PCR screening of the recombinant plasmid: analysed by electrophoresis on a 3% agarose gel. Lanes: 1. pBR322 cut with HpaII; 2. pMAL-pk negative control (113 bp); 3-7. colonies on a pMAL-pk ligation plate; 8. pMAL-ck negative control (113 bp); 9-10. colonies on a pMAL-ck ligation plate.

3.3 Discussion and summary

The ligation of two blunt-ended DNA fragments, was less efficient than the ligation of DNA fragments either with one blunt-end and one sticky-end, or two sticky ends [46]. Therefore, the experimental design of the insertion reaction had to take the difficulty of ligation into account.

Accordingly, a synthetic gene sequence encoding an enterokinase recognition site (pentapeptide) was ligated into the pMAL vectors because enterokinase was planned to be used to release the expressed systemin from a MBP-fusion protein. These modified plasmids were called pMAL-pk and pMAL-ck.

After the pMAL-pk/ck plasmids were linearised by EcoRI, nuclease P1 was used to remove the four overhanging bases at the 3' ends to produce the blunt ends. The choice of the correct concentration of nuclease P1 in the reaction solution and the precise incubation time was crucial for accuracy of the digestion.

As it turned out, only one pMAL-pk plasmid was ligated in such a manner that the systemin gene was both complete and correctly orientated. The colony which contained the recombinant plasmid was amplified to express systemin in *E. coli* cells.

Chapter 4 Expression, isolation, and purification of recombinant systemin

4.0 Contents

4.1 Introduction

The systemin gene was ligated to the *malE* gene in the pMAL-pk plasmid and the plasmid was transformed into *E. coli* cells. Systemin was thus expressed with the maltose-binding protein as a fusion protein. This fusion protein could be separated from other bacterial proteins by using an affinity column. The size and purity of this fusion protein was examined by SDS polyacrylamide gel electrophoresis (PAGE).

The fusion protein was digested by enterokinase at optimal temperature and enzyme concentration. The digested samples were examined either on an SDS gel or on HPLC. A special discontinuous gel system [67] could be used for the separation of low molecular weight peptides but results were found to be inconsistent. However, high-performance liquid chromatography (HPLC) is a more suitable method than gel electrophoresis for detecting the presence of small peptides.

Recombinant systemin peptide was separated from the mixture of fragments in the digestion pool by using HPLC. The different fractions were collected. The fractions of interest were sequenced on a gas-phase peptide sequencer.

4.2 Purification of MBP-systemin fusion protein

In the pMAL system, the *tac* promoter which controlled the *malE* gene was activated through IPTG induction. 1 ml of the overnight *E. coli* cells (containing recombinant pMAL plasmid) was inoculated into 1 litre LB medium (Appendix). The *E. coli* cells were incubated at 37°C until the desired cell concentration was reached. IPTG was then added to the medium. One hour later, *malE* fusion protein was expected to be produced as an extra cell protein. The occurrence of the new band was examined on an SDS gel. The *E. coli* cells were harvested 3 hours after induction.

Not knowing the location of the fusion protein in the cells, two protein extraction methods were used: (a) the cold osmotic shock method which isolates any bacterial periplasm proteins, and (b) the sonication method which extracts the total cell proteins [Manual C].

The osmotic shock method is commonly employed to release periplasmic proteins [52]. It involves the pre-treatment of cells with a concentrated sucrose solution and EDTA followed by a rapid dilution into a medium of low osmotic strength. Periplasmic proteins are expelled during this procedure by the sudden expansion of the inner membrane against the cell wall. The cells are then removed from the resulting periplasmic fraction by centrifugation [52].

Ultrasonication on the other hand breaks down the bacterial cell walls. This method therefore releases all the cell's proteins. The soluble and insoluble proteins are then separated by centrifugation. The fusion protein appears either with the soluble or with the insoluble proteins depending on its hydrophilicity [Manual C].

Having collected the total cell proteins, and the periplasmic space proteins, by these methods, each fraction was separately loaded onto a crosslinked amylose resin as the affinity matrix to purify the MBP fusion protein. The MBP fusion protein binds to the resin [Manual C]. The

unbound proteins were washed off from the column by washing buffer. Later, the fusion protein was eluted with column buffer which contains maltose (Fig. 4.1).

The results of the pilot experiment indicated that the MBP-systemin fusion protein was mostly secreted into the periplasm because of the presence of the leading sequence in the MBP. The cold osmotic shock method was used to extract total periplasmic space proteins. The different protein fractions were loaded onto an SDS gel (Fig. 4.2).

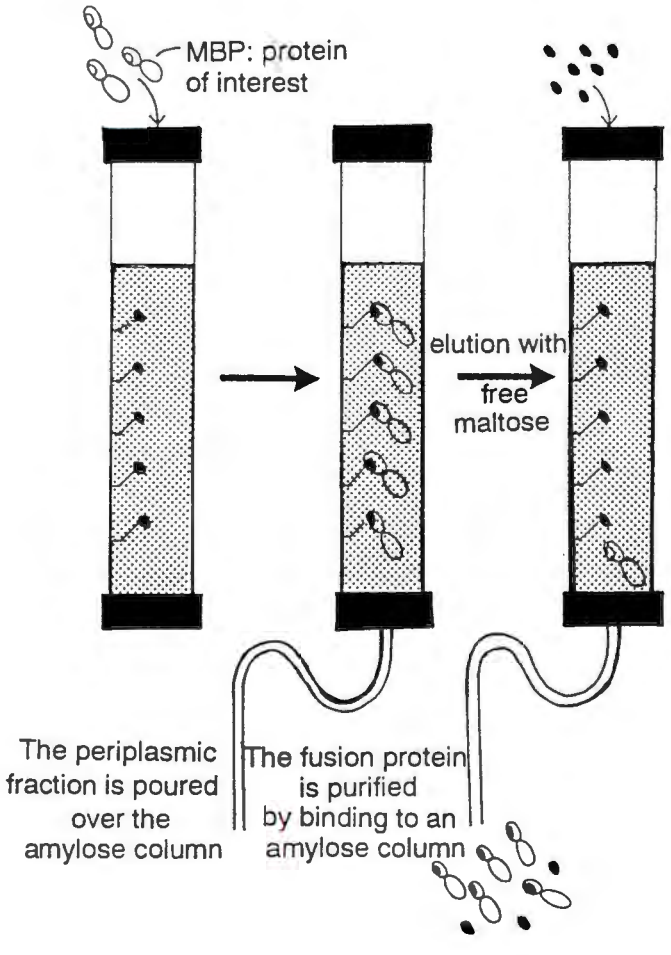


Fig. 4.1 The procedures of purification of MBP-systemin fusion protein. The cell extract was loaded onto a column of amylose resin. The MBP fusion protein bound to the column and the remaining proteins in the extract were washed through the column. The fusion protein was eluted with free maltose and then cleaved with enterokinase to release systemin from MBP.

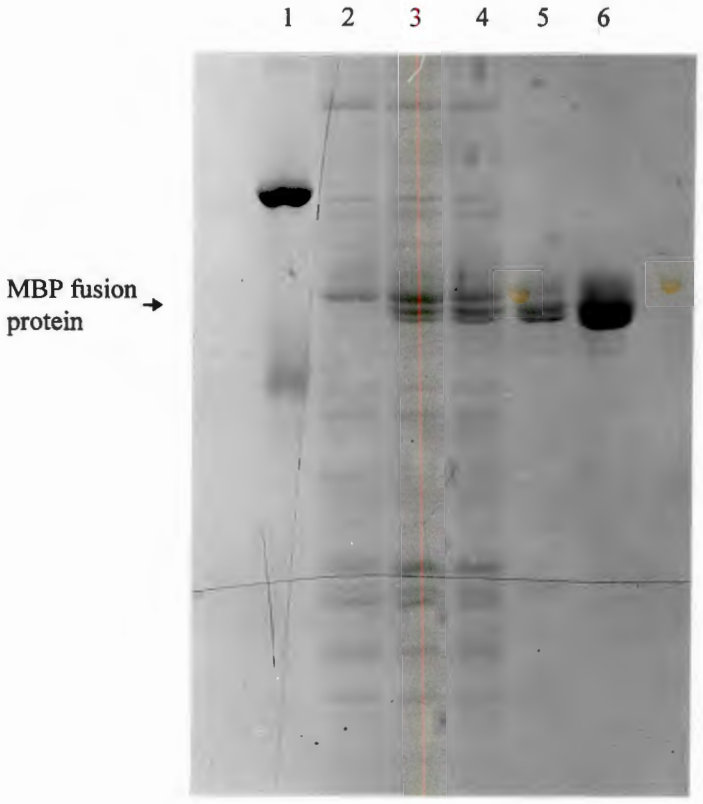


Fig. 4.2 SDS-electrophoresis of fractions collected in the different expression stages. Lanes: 1. protein molecular weight marker; 2. 0 h induction; 3. 2 h induction; 4. 3 h induction; 5. total periplasmic space protein; 6. proteins eluted from the affinity column by elution buffer.

4.3 Digestion of the fusion protein

We found that the undenatured fusion protein could not be cut with enterokinase, presumably because the protease site was not accessible. We had to denature the fusion protein to enhance the cleavage. The fusion protein was dialysed against 6 M guanidine hydrochloride and then desalted by dialysis against Tris buffer. It is believed that after denaturation and dialysing out the denaturant, protein folding occurs either differently or slowly enough to allow cleavage to take place [60]. The denatured protein was lyophilised in Tris buffer. The protein pellet was redissolved in H_2O and diluted to a final concentration of 2 mg/ml. For good enterokinase digestion, the fusion protein concentration was kept at 2 mg/ml in 10 mM Tris·HCl buffer, pH 8.0 [40].

20 units of enterokinase were used for the digestion of every 100 mg denatured fusion protein. The reaction was incubated at 42°C for 48 to 72 hours. 5 µl aliquots were collected from the digestion mixture every 24 hours. The results are shown in Fig. 4.3.

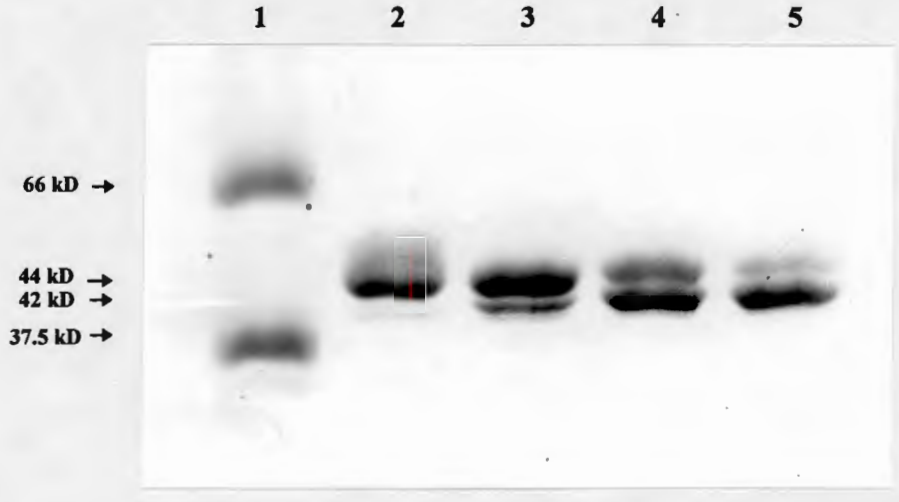


Fig. 4.3 SDS-polyacrylamide gel electrophoresis of MBP fusion protein following limited proteolysis with enterokinase. Lanes: 1. molecular weight marker; 2. 0 h; 3. 24 h; 4. 48 h; 5. 72 h of digestion.

The size of the protein was changed from 44 kDa to 42 kDa through digestion. The ratio of the intensity of the 44 kDa to 42 kDa band was decreased with increasing time.

Samples from the digestion were also injected onto a reverse phase HPLC C18 column (4.6 mm × 15 cm, TSK-gel ODS-80TM, TosoHaas). Solvent A consisted of 0.1% TFA in 100% H_2O, solvent B consisted of 0.1% TFA in 60% acetonitrile, 40% H_2O. To prevent

contamination and for degassing, both solvents were filtered through a 0.22 μm Millipore filter. One of the HPLC profiles is shown in Fig. 4.4.

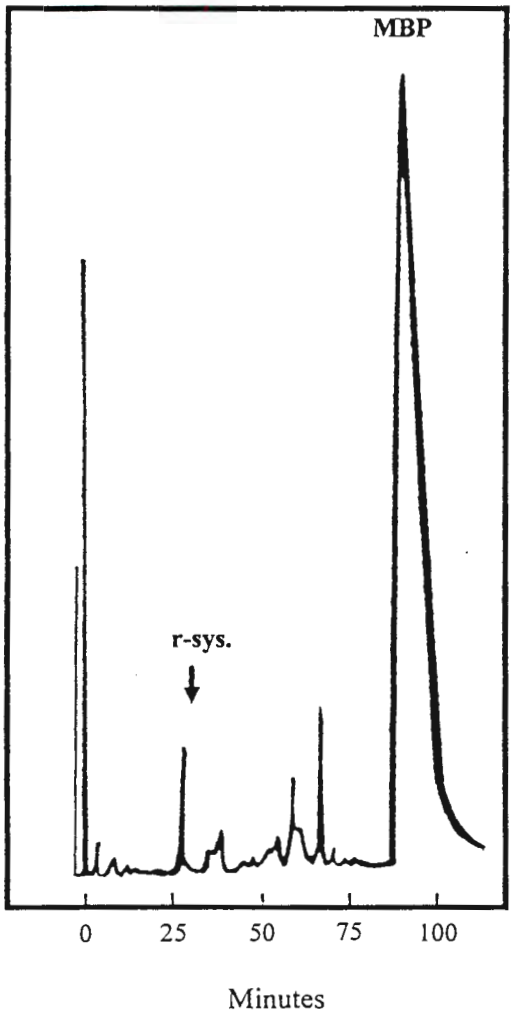


Fig. 4.4 HPLC profile of the digested sample. The flow rate was 1 ml/min and eluted peaks were monitored at A_{210}. A linear gradient of 100% A to 100% B over 120 minutes was used.

The HPLC profile of the sample containing the fusion protein only showed a single peak after 89 minutes (44% acetonitrile). The fusion protein (MBP-systemin) and the native MBP could not be separated on HPLC. The slight differences of size and hydrophilicity were not significant enough for MBP and the fusion protein to be separated (Table 4.1). A peak eluting after 30 minutes (15% acetonitrile) only appeared in the sample that had been digested.

Table 4.1 The different physical characteristics of systemin, MBP and MBP-systemin fusion protein molecules.

	Systemin	**MBP**	**MBP-systemin**
Residues	18	389	407
Molecular weight	2010	42527	44519
Hydrophobic A.A.	61.6%	43.7%	44.5%
Charge	2	-12	-10
Isoelectric point	10.49	4.82	4.96

4.4 Purification of recombinant systemin

The fraction eluting after 30 minutes (15% acetonitrile) was collected. The peptide was sequenced on a gas-phase peptide sequencer (Chapter 5)[33]. The first 5 amino acids from the N-terminus were compared with the N-terminal sequence of native systemin.

Once the systemin purification procedure was established, a large batch of fusion protein was digested and systemin peptide was isolated and purified by HPLC as described.

4.5 Discussion and summary

In the pMAL-pk expression system, the fusion protein was secreted into the *E. coli* periplasmic space. The cold osmotic shock method, which allows one to harvest the periplasmic space proteins was used to obtain the fusion protein. This method was quick and

simple with little contamination by other proteins. The fusion protein was separated from other periplasmic space proteins by affinity column chromatography. On an SDS gel, the fusion protein shows only one band. The affinity-purified protein was considered pure. The yield of fusion protein was about 12-15 mg/l *E. coli* cells in the LB medium. Theoretically, 0.68 mg of systemin peptide could be harvested from a 1 litre cell culture.

The pure fusion protein was denatured in a 6 M guanidine hydrochloride solution. The denatured protein was then dialysed against the Tris buffer to remove guanidine hydrochloride. The protein concentration was adjusted for the enterokinase digestion.

When the concentration of enterokinase in the enzyme/protein mixture was too high, the SDS gel which was used to identify the proteins showed multiple bands. Non-specific digestion obviously occurred. The HPLC profile supported this finding (results not shown). Under these conditions, it was difficult to isolate systemin from other proteins.

There was only one peak on the HPLC profile when non-digested fusion protein was injected onto the HPLC. A new peak representing a protein which was less hydrophobic than MBP was detected in the digested sample. The retention time (15% acetonitrile) of the new peak was very close to the expected systemin elution time (14.3-15.3% acetonitrile) as reported by Pearce [55]. MBP contained an average of 43.7% hydrophilic amino acids and systemin contained 61.6% hydrophilic amino acids in their total amino acid composition. The HPLC results were in good agreement with the calculated hydrophilicity.

The material contributing to the new peak (eluted at 15% acetonitrile) was collected for further analysis as described in the following chapter.

Chapter 5 The physical characteristics of recombinant systemin

5.0 Contents

5.1 Introduction

The recombinant systemin peptide was purified by using HPLC. Its physical characteristics, including the amino acid composition, amino acid sequence, HPLC retention time, and molecular weight were determined. A commercially synthesised systemin peptide was used as a standard.

5.2 Amino acid analysis

Two approaches are routinely used for amino acid analysis. The first approach uses classical ion exchange chromatography with post-column derivatisation. This method has been improved by Klapper [39]. He applied the modern gradient HPLC instrumentation to obtain rapid and high resolution analysis. The second approach is based on reverse-phase separation with pre-column derivatisation.

As it turned out, the first approach is the method of choice for the amino acid analysis of r-systemin. The experimental protocol of the post-column derivatisation approach works as follows: the peptide to be analysed is hydrolysed by the acid in the gas-phase, for instance,

6 M HCl. After hydrolysis, the acid is evaporated. The amino acids are redissolved in HPLC solvent A and loaded onto an ion-exchange column. The eluting amino acids are then reacted with orthophthaldehyde (OPA) for post-column flourescence detection. Alternatively, colorimetric detection can be accomplished by reaction with ninhydrin [39].

The separation is based on ionic interaction of the hydrolysate with a strongly acidic resin as well as secondary hydrophobic interactions with the polymer backbone. The acidic amino acids will have the weakest interaction and elute first. They are followed by the hydroxylated, the neutral, and finally by the basic amino acids. This complex separation requires an increase in the either pH or the ionic strength of the elution buffer [39].

A pH gradient elution at a constant sodium ion concentration of 0.2 M was used. Solvent A consists of sodium citrate, pH 3.25 and solvent B consists of sodium borate, pH 9.5. The linear gradient changes from 100% A to 100% B over 90 minutes. The result of the amino acid analysis is shown in Table 5.1.

Table 5.1 The amino acid composition of systemin.

	D	**T**	**S**	**E**	**A**	**V**	**M**	**K**	**R**	**P**
Amino acid yield	2	1	2	2	1	1	1	3	1	2
r-Systemin (nanomole)	0.58	0.29	0.69	0.47	0.32	0.35	0.21	0.87	0.20	-
s-Systemin (nanomole)	0.67	0.30	0.53	0.69	0.30	0.34	0.34	0.91	0.29	-

5.3 Peptide sequencing

5.3.1 The principles of peptide sequencing

We used the Edman degradation method for sequence determination [33]. The chemical process is a repetitive reaction to generate the amino acid sequence starting from the N-terminus. The free α-amino group of the polypeptide chain is coupled to phenylisothiocyanate under alkaline conditions which allows the terminal amino acid to be cleaved from the remainder of the peptide with acid. The resulting amino acid derivative (an anilinothiazolinone) is converted into the more stable phenylthiohydantoin (PTH) amino acid, which is analysed by HPLC. The remaining peptide is subjected to further degradation cycles [33]. All the reactions are carried out on an automated gas-phase protein/peptide sequencer.

Before starting the sequencing procedures, the peptide sample is embedded in Polybrene (a polymeric quarternary ammonium salt that adheres strongly to both glass surfaces and peptides). The sample is then dried onto a porous glass fibre disc and transferred into a small cartridge-style reaction cell. Now the peptide is non-covalently attached to the support. Polybrene prevents the sample from being dislodged by solvents flowing through the column.

5.3.2 The repetitive yield of peptide sequencing

The steady decrease of the signal during a sequencing experiment is due to a number of factors: physical loss of sample in the reaction vessel; side reactions that block a portion of the amino-terminal residue at each cycle; acid-catalysed cleavage of the peptide chain; and incomplete coupling or cleavage that results in carryover of amino acids into subsequent cycles [33].

The overall efficiency, limited by these losses, is expressed by the repetitive yield (R.Y.), defined as

$$R.Y.= (Yn/Y1)^{1/n-1}$$

where Yn = yield at cycle n; Y1 = yield at cycle 1.

5.3.3 The sequencing result of recombinant systemin

About 800 ng of r-systemin was loaded onto the sequencer. The first fifteen amino acids from the N-terminus were sequenced. The yield of every cycle is shown in Table 5.3.

5.4 HPLC separation

A known amount of synthetic systemin was loaded onto a reverse phase HPLC C18 column and analysed under the same separation conditions as for recombinant systemin purification (Chapter 4), except that the gradient times were increased (Fig. 5.1). The peak area of s-systemin was compared to the peak area of the unknown amount of r-systemin to estimate the concentration of r-systemin.

Synthetic and recombinant systemin (about 100 to 250 ng each) were run separately on the HPLC as described above. The two samples were then mixed together for the third HPLC run. If they both had the same hydrophilicity, the peptide peaks were expected to superimpose.

Table 5.2 R.T. and peak areas of the three HPLC experiments (compare with Fig. 5.1).

Samples	**S-systemin**	**R-systemin**	**R+S systemin**
Retention time (min)	26.886	26.886	26.913
Peak area	7360	3962	12269

Table 5.3 The peptide sequencing results from N-terminus to C-terminus.

Cycle (N→C)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Published sequence	A	V	Q	S	K	P	P	S	K	R	D	P	P	K	M
Sequencing result	A	V	Q	S	K	P	P	S	K	-	D	P	P	K	M
Yield (picomole)	403	292	341	79	130	145	132	47	50	-	37	42	59	9	15

The R.Y. was about 79%.

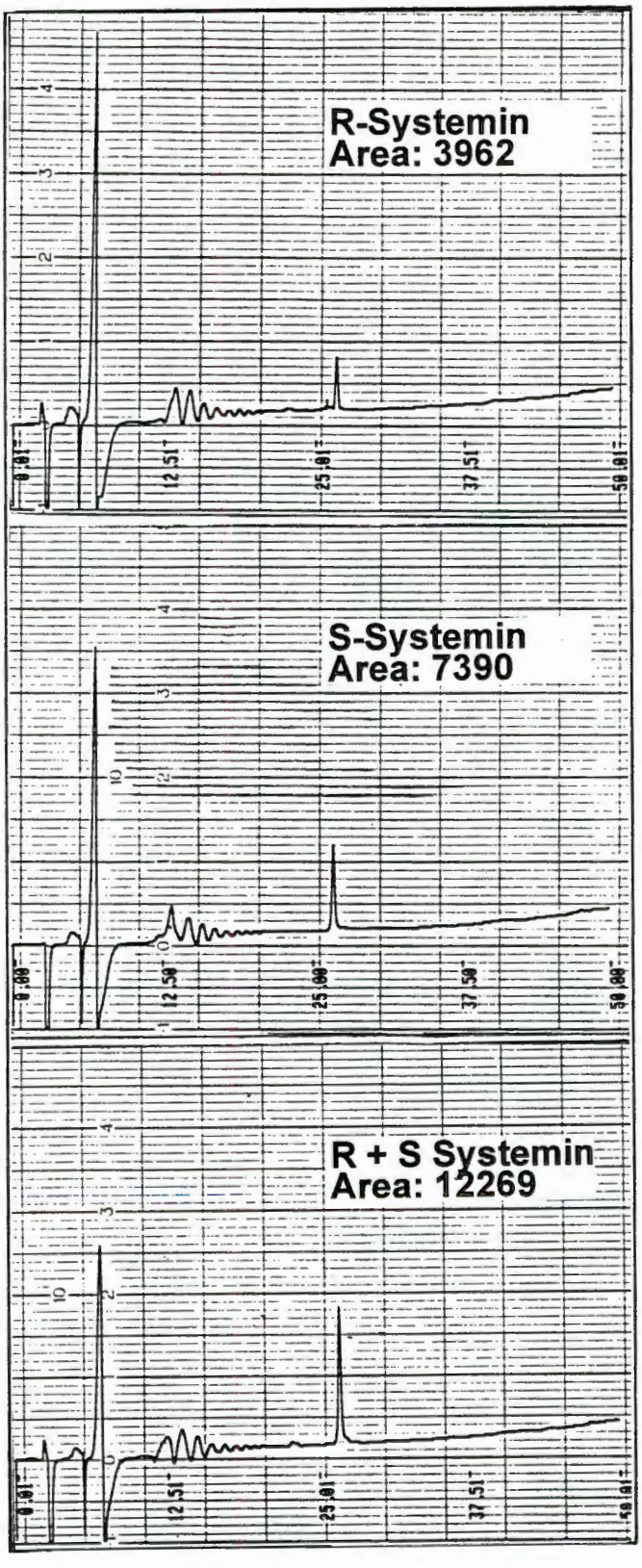


Fig. 5.1 Retention times of three HPLC runs. A linear gradient was run from 100% solvent A to 50% solvent B in 50 minutes. Solvent A contained 0.1% TFA in 100% H_2O, solvent B contained 0.1% TFA in 60% Acetonitrile, 40% H_2O.

5.5 Mass spectrometry of r-systemin

The recombinant systemin was analysed on a Kratos KOMPACT MALDI III (matrix assisted laser desorption/ionisation time-of-flight mass spectrometer). The instrument was first calibrated in both linear and reflection mode using mellitin (0.5 µl of 1 pmol/ml) as an external standard. Optionally, mellitin was present in the sample to provide an internal calibration.

Subsequently, the peptide was analysed in each mode. 0.5 µl of r-systemin solution (15 pmole) was deposited on the sample slide together with 0.5 µl of the laser-absorbing matrix solution (α-cyano-4-hydroxycinnamic acid). Each spectrum was the sum of 50 laser shots and took between 20-25 seconds (Fig. 5.2).

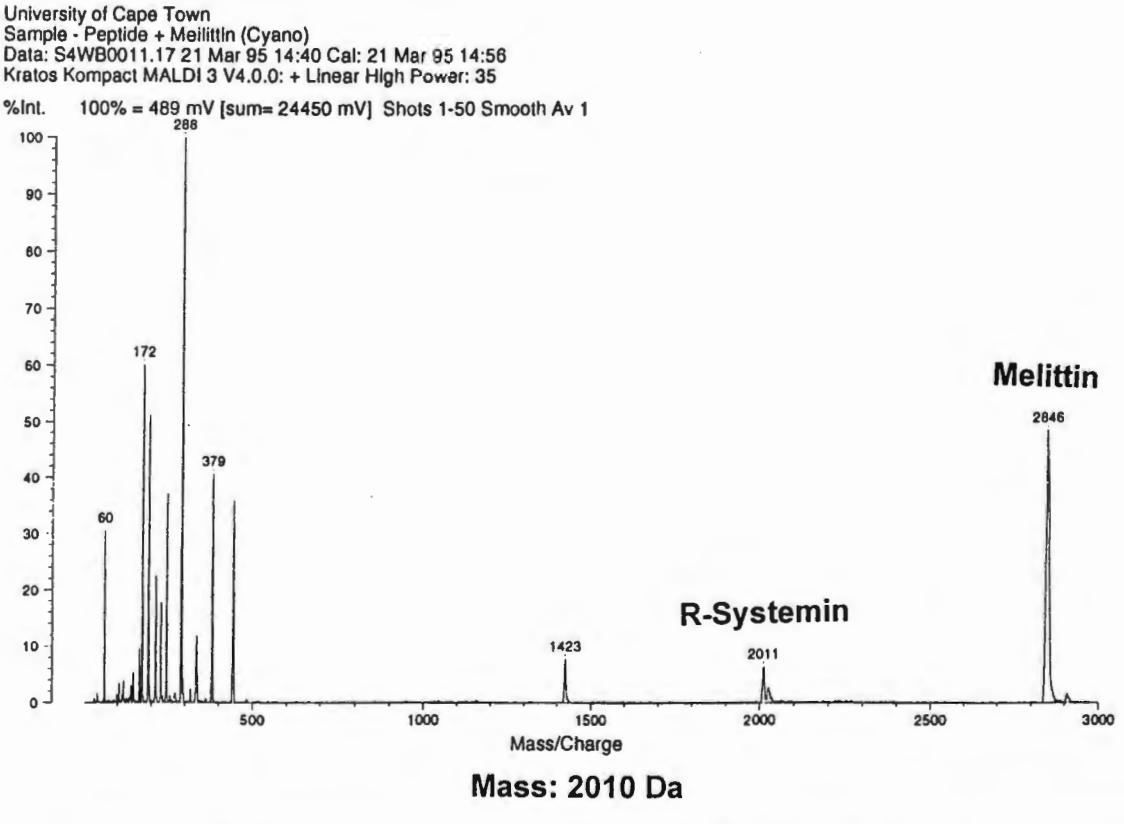


Fig. 5.2 The result of the mass spectrometry of r-systemin. The data indicated a mass of 2011 Da for r-systemin. The calibrated actual mass of the peptide would be 2010 Da. The published molecular weight of systemin is 2010.3 [55].

5.6 Discussion and summary

The amino acid composition results of s-systemin and r-systemin have been compared. Data for proline in the sequence are missing due to the inability of the OPA system to detect proline and hydroxyproline. An oxidising reagent such as sodium hypochlorite must be mixed with the column eluent before the OPA reagent is added in order to render the amino acids reactive to OPA [Manual B].

R-systemin was sequenced on a gas-phase sequencer. The sequencing result on the 10th amino acid from the N-terminus (arginine) was lost inadvertently. The yield dropped dramatically after every serine cycle. The R.Y. (~80%) was also lower than for the normal protein sequencing on the same sequencer (90-95%).

However, the sequence of the peptide being analysed is crucial. Peptides with a high content of serine, threonine, or aspartic acid can undergo acid-catalysed cleavage at these residues during the TFA delivery step in the sequencing cycle, resulting in a large background level of all PTHs and a loss of signal when these positions are reached [29]. This factor might explain the decreasing yield in every cycle of sequencing when serine is cleaved (Table 5.3).

The poor repetitive yield may be due to several influences. For example, the R.Y. is generally lower on sequencing short peptides than on sequencing proteins because of the higher physical loss of sample from the filter disc. Short peptides can have significant solubility in the extraction solvents, and these peptides often show very little repetitive yields, especially near their C-termini [29].

The retention times of both r-systemin and s-systemin were identical. The two peaks overlapped when the two peptides were mixed. This result indicated the similarity of their hydrophilicity. The sum of s-systemin and r-systemin added from the individual samples

differs from the sum obtained from the mixture by 8.4%. Incorrect sampling and the loss during HPLC runs could possibly account for the difference.

The r-systemin molecular weight obtained from mass spectrometry was identical with the published result. Hence, we can conclude that no amino acid modification occurred during the isolation and purification procedures. The r-systemin was subsequently tested *in vivo*.

Chapter 6 Test of the biological function of recombinant systemin

6.0 Contents

6.1 Introduction

Chapter 5 dealt with establishing the physical characteristics of r-systemin, and compared them to s-systemin. The next step was to determine the biological functioning of r-systemin.

It is known that native systemin, synthetic systemin, and wounding induces the accumulation of proteinase inhibitor I (PI-I) in young tomato plants [55]. The question is, can recombinant systemin induce the same response, namely an increase of PI-I level? If this response can be shown, then r-systemin could be substituted for native systemin in other *in vivo* experiments.

To record the accumulation of PI-I, the level of PI-I mRNA was determined after wounding the leaves or applying r-systemin to the plant through a cut stem.

In order to examine the changes in PI-I levels, we generated a specific probe for PI-I mRNA. The probe was synthesised by PCR amplification of a part of the PI-I gene. This amplified DNA sequence was cloned into a pBluescript plasmid.

Twenty-one to twenty-eight days old tomato plants (*Lycopersicon esculentum* FMTT 22) were used for the *in vivo* tests. The plants were grown in a greenhouse (the seeds were a gift from Mr. L.M. Engle, The Asian Vegetable Research and Development Centre, Taiwan, R.O.C.).

Polyclonal antibodies against the MBP-systemin fusion protein generated in a rabbit were used for the western blot experiments. These antibodies were examined for their binding efficiency to r-systemin and s-systemin. The procedures for the biological activity test are shown in Fig. 6.1.

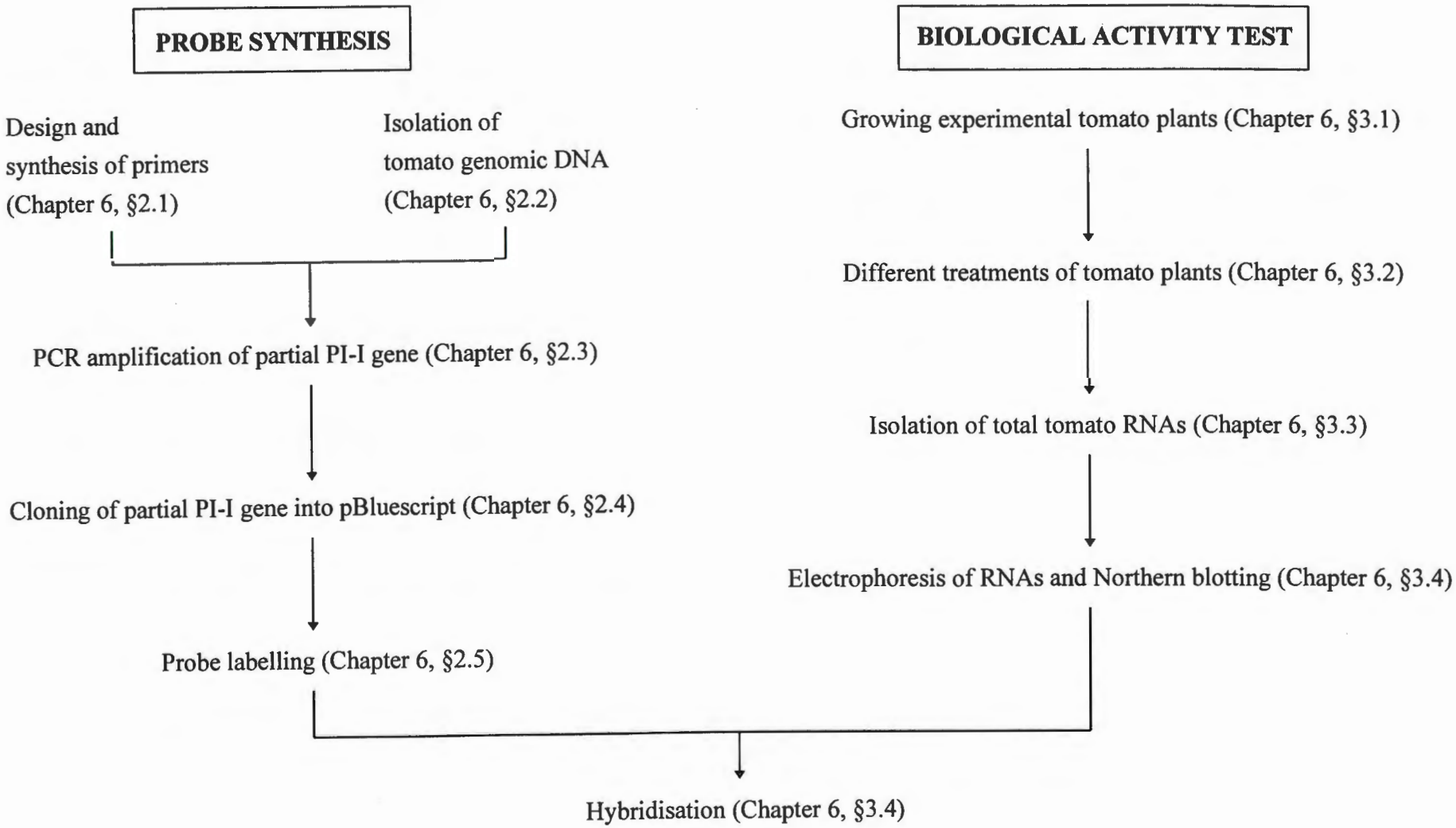


Fig. 6.1 Flow chart for the biological activity test.

6.2 Synthesis of the proteinase inhibitor I probe

6.2.1 Primer design

Two PCR primers were designed based on the known PI-I DNA sequence for PCR amplification [29]. The gene sequence to be amplified was part of the open reading frame which could hybridise to the mRNA (Fig. 6.2). The primers were 29 and 30 bases long respectively. Their annealing temperatures were calculated using the "primer" programme of GCG. The estimated annealing temperatures were both between 57°C and 58°C. An extra restriction site sequence (EcoRI) was added to the 5' end of the forward primer and a SalI site sequence was added to the 5' end of the reverse primer. They were designed for ligation of the PCR product into the pBluescript plasmid.

The sequences of the two primers are as follows:

Forward primer: 5'CA**GAATTC**CTGCAACTCCCATTTTTAAAA 3'
EcoRI site

Reverse primer: 5'TG**GTCGAC**TGTGCCTGCTTAATTACTTCAA 3'
SalI site

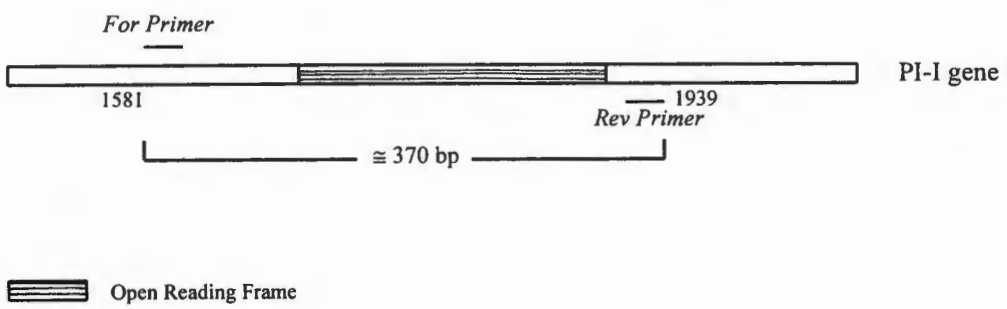


Fig. 6.2 The PCR amplification of part of the PI-I gene.

6.2.2 Isolation of total tomato genomic DNA

A one-step plant genomic DNA isolation method was used to extract total tomato genomic DNA for PCR (Chapter 8)[5]. Generally, 25-35 μg total genomic DNA can be extracted from two small discs of tomato leaf (1 cm in diameter each). The extract was examined by agarose gel electrophoresis.

6.2.3 PCR amplification

The partial PI- I gene sequence for cloning was amplified by PCR. The reaction mixture contained dNTPs, DNA polymerase, polymerase buffer, primers and total tomato genomic DNA (template). The template concentration was optimised.

The cycle profile of PCR was: step 1, 90 sec at 94 °C; step 2, 20 sec at 94 °C; step 3, 30 sec at 57°C; step 4, 45 sec at 72°C; and step 5, 180 sec at 72°C. The cycle was repeated 30 times from step 2 to step 4. The PCR products were then loaded onto a non-denaturing acrylamide gel. A molecule weight marker was also run.

6.2.4 Cloning and sequencing

According to the choice of the primers on the PI-I gene, the PCR product was expected to be 374 bp in length. A PCR product of this size was obtained. Subsequently this PCR product was double digested by EcoRI and SalI at 37°C for 2 hours. The digested DNA fragment was desalted by a single ethanol precipitation step before ligation into pBluescript.

Details of the cloning procedure are described in Chapter 3. The amplified pBluescript plasmid was isolated from the *E. coli* cells by using a large scale plasmid preparation method (Chapter 8). The plasmid was digested with the enzymes, EcoRI and SalI .

The ligation reaction vials were incubated in an ice water bath. The temperature of the bath increased from 2-16°C during overnight incubation. The ligated samples were transformed into competent *E. coli* cells (XL-B). The transformation results are shown in Table 6.1.

Table 6.1 The results of the transformation.

Ligation samples	**Colony number**
H_2O only	0
Supercoiled plasmid	3480
CIP linear plasmid	0
CIP linear plasmid with ligase (low conc. ligase)	0
CIP linear plasmid + insert with ligase (low conc. ligase)	4
CIP linear plasmid + insert with ligase (high conc. ligase)	15

0.02 pmole of plasmid and 0.02 pmole of insert were ligated and transformed.

Small quantities of plasmid were prepared from thirteen colonies. The colonies were selected from different plates including two negative control samples (non-recombinant plasmid). The following restriction digestion mapping method was used to screen the colonies. Each plasmid sample was double digested by EcoRI and SalI. If the plasmid contained the insert, one extra band (the insert, 370 bp) should be released from the plasmid. Otherwise, the plasmid would only be linearized. The digested samples were analysed by electrophoresis on a 1.5% agarose gel.

Two out of the eleven isolates showed an extra band on the gel after digestion. The plasmids were then sequenced. 180 bases were read from a pBluescript reverse primer and 198 bases were read from a forward primer. The results were compared with the known PI-I gene sequence.

6.2.5 Labelling of the probe

A PCR labelling method was used to incorporate the non-radioactive DIG-11-dUTP (digoxigenin) into the DNA product [Manual B]. The recombinant pBluescript plasmid was used as a template. The primers were the same as those used to amplify the PI-I gene. A ratio of 35% DIG-11-dUTP and 65% dTTP (1:2 mix) in the labelling mixture was suggested by the protocol because it yielded the most sensitive probes for filter hybridisation [Manual B].

The PCR product containing DIG-dUTP was larger than the unlabelled PCR product. The larger DIG-labelled DNA was retarded in its movement through an agarose gel [Manual B]. The successfully labelled PCR product could be compared with an unlabelled PCR product on an agarose gel. An alternative assay for successful labelling was to examine the PCR product on a nylon membrane. 1 µl of labelled and 1 µl of unlabelled DNA were loaded on a piece of nylon membrane and dried. The membrane was treated by following the DIG detection procedures to visualise the labelled sequences (Chapter 8). The DIG-labelled PCR product showed a black spot when the membrane was exposed to an X-ray film.

6.3 Activity test of r-systemin

6.3.1 Tomato plants

The tomato plants were grown in two different environments to determine the optimum conditions for growing the experimental plants. One batch was grown inside a room equipped with temperature control (22-26°C) and artificial lights (light cycle: 12 h light, 12 h dark). The other batch was grown in a greenhouse with natural sunlight and an average temperature of 22-28°C. Three different cultivars of tomato were tested. They were *Lycopersicon peruvianum*, *Lycopersicon esculentum* Heinz and FMTT 22. Best results were obtained with *L. esculentum* FMTT 22 grown in the greenhouse.

6.3.2 Experimental design for the activity test

Pilot experiment

R-systemin was applied to the plants through cut stem in order to examine its biological function. Pearce suggested [55] that in order to avoid the initiation of natural wounding response by cutting, a sharp razor blade should be used to cut the stems with care. In a pilot experiment we determined the basic PI-I mRNA levels in leaves of unwounded plants which were cut from their stem bases and incubated for a period of time.

Three groups of young tomato plants (3 plants per group) were carefully cut off as described and incubated in small containers with distilled water for 6 hours. The three groups were labelled "unwounded, 0 h", "unwounded, 6 h", and "wounded, 6 h". In the "wounded" groups, the main veins of the two lower leaves of each plant were pressed by a haemostat two or three times (Fig. 6.3).

Fig. 6.3 The three groups in the pilot experiment.

In order to obtain the original PI-I level in each plant of the wounded groups, one leaf was taken at 0 h after wounding, another one was taken 6 h later. In the unwounded group (0 h), one leaf from each plant was taken immediately when the plants were cut off. The three plants from "unwounded, 6 h" group were kept in water for 6 h after being cut off and then one leaf from each plant was taken. The total RNA from each leaf sample was extracted for the northern blotting experiments.

Systemin bioassay

Based on the results of the pilot experiment, five groups of plants were tested in the systemin induction experiment. They were: (A) unwounded, 0 h; (B) unwounded, 6 h; (C) wounded, 6 h; (D) s-systemin treated, 6 h; and (E) r-systemin treated, 6 h, five plants per group. The plants were grown and tested in a greenhouse.

The tomato plants were cut off at their stem bases. The cut stems were first soaked in small vials with 1 ml, 10 mM sodium phosphate buffer, pH 6.5 [55]. In groups D and E, 100 fmole of s- or r-systemin respectively were added into the above buffer. The buffer of the other three groups did not contain any systemin. After 40 to 60 minutes, the plants were then transferred to larger containers filled with distilled water. One leaf from each plant of group A was collected immediately after transfer. One leaf from each plant in the group B, C, D, and E was taken after 6 h incubation. All the leaves were kept frozen in liquid nitrogen until the total RNA had been prepared. During incubation, all the plants were protected from insect attack by keeping them in a wooden framed box covered with fine mesh. The light source was natural sunlight. The temperature varied between 22–28°C (Fig. 6.4).

Fig. 6.4 The experimental environment.

6.3.3 Isolation of total RNA

The RNA isolation method of Verwoerd [77] was modified to extract the RNA from the tomato leaf samples (Chapter 8). All the reagents and instruments which had direct contact with RNA samples were kept RNase free [1]. The concentration of total RNA was determined spectrophotometrically (1 OD at 260 nm is equal to 40 μg of RNA)[1]. The yield of total RNA from every 100 mg leaf tissue varied between 50 and 70 μg. The extracted RNA samples were examined on an RNA agarose gel system [85].

6.3.4 Northern blotting and hybridisation

The total RNA samples were separated by electrophoresis on a 1.7% RNA agarose gel under denaturing conditions (Chapter 8)[85]. The RNA bands were blotted onto a N^+-nylon membrane (Amersham) by capillary force. The RNAs were cross-linked onto the membrane by exposing to a UV lamp [Manual A].

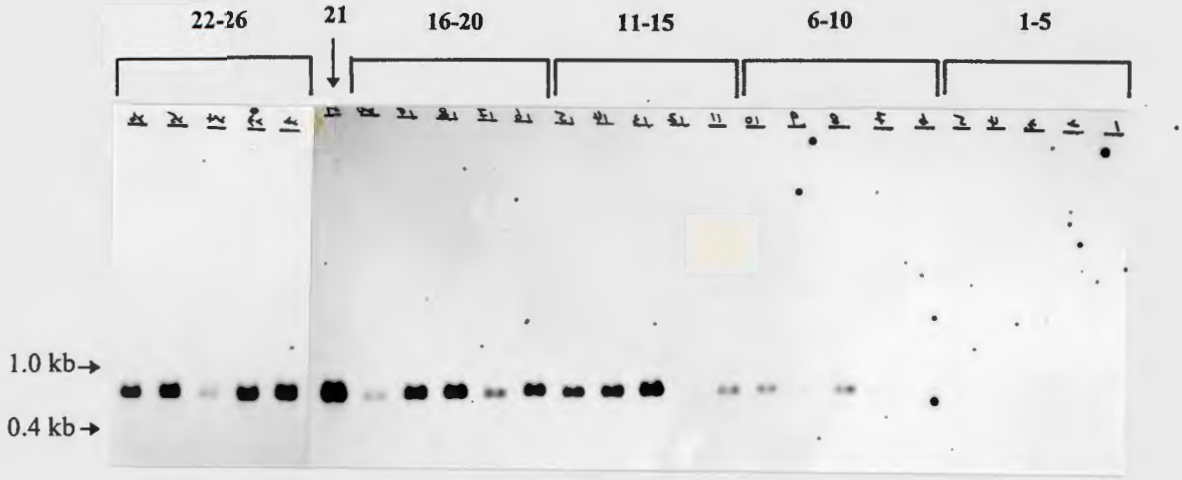
For prehybridisation, the membrane was placed in a glass tube with prehybridisation solution and incubated at 42°C for 1 to 2 hours in a hybridisation oven [Manual B]. The hybridisation solution was the same as that used for the prehybridisation procedure, but with labelled single-stranded probe. The glass tube containing hybridisation solution was incubated at 50°C overnight on a low speed roller [Manual D].

Excess or non-specifically bound probe on the membrane was washed off with SDS in diluted SSC buffers. The washing stringency depended on the concentration of buffer and the washing temperature: the higher the salt concentration and the lower the temperature, the lower the washing stringency [Manual A].

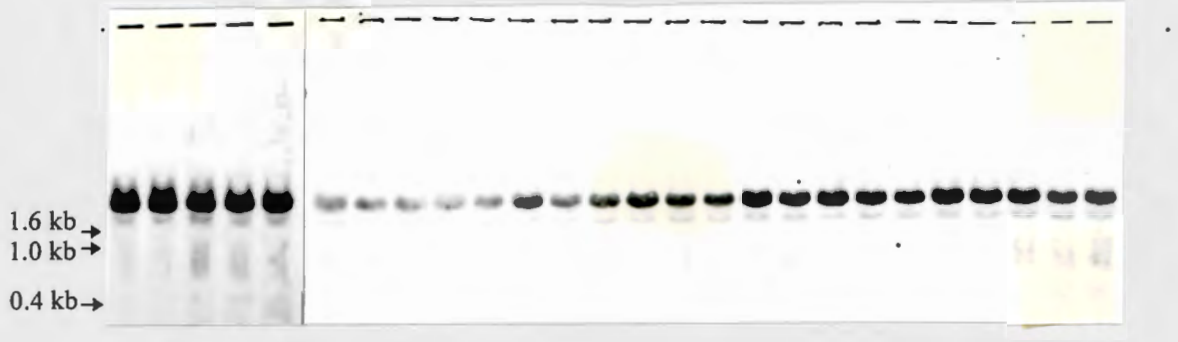
The DIG label in the probe which had hybridised to the RNA was immuno-detected with an anti-DIG-alkaline phosphatase. The alkaline phosphatase reacted with the chemiluminescent alkaline substrate and produced a light signal which was detected by exposing the membrane to an X-ray film. The exposure time depended on the intensity of the signals (Chapter 8).

6.3.5 Analysis of biological function test

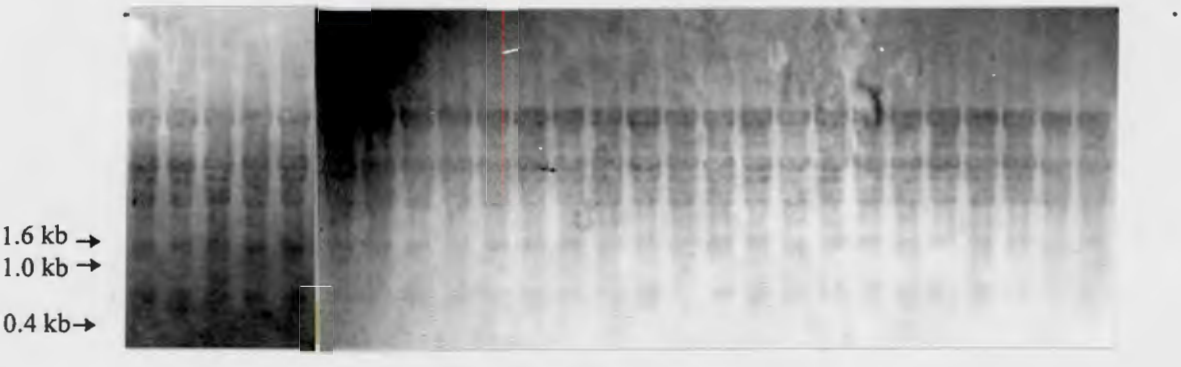
The hybridisation results of the pilot experiment and of the systemin induction test are shown in Fig. 6.5. The X-ray film was scanned by a densitometer (GS 300-Hoefer Scientific Instrument) and the band density was digitalised in order to calculate the changes of PI-I mRNA level.



A. PI-I partial gene probe.



B. 18S rRNA probe.



C. Membrane stained with methylene blue.

Fig. 6.5 Hybridisation results, using PI-I mRNA probe (A) or 18S rRNA probe (B), and the result of membrane staining (C). Lanes: 1-5. unwounded, 0 h; 6-10. unwounded, 6 h; 11-15. wounded, 6 h; 16-20. s-systemin applied; 21. positive control from a previous experiment; 22-26. r-systemin applied. (The size of PI-I mRNA is 511 bp.)

To examine the amounts of RNA samples loaded, the PI-I probe on the membranes was stripped off and the membrane was reprobed with a DIG-labelled 18S rRNA oligonucleotide probe. The intensity of the bands on the X-ray film could be quantified with a scanner. The ratio of the PI-I mRNA and 18S rRNA signal was calculated (Table 6.2). The statistical result is shown in Fig. 6.6 [86].

The quantity of the RNA samples bound to the membranes was also examined by staining the membrane with a methylene blue staining solution. This staining helps to determine the quantity or quality of the RNA immobilised on the hybridisation membranes (Fig. 6.5).

Table 6.2 Results of systemin inducing experiments.

	Unwounded 0h area ratio	**Unwounded 6h area ratio**	**Wounded 6h area ratio**	**S-systemin 6h area ratio**	**R-systemin 6h area ratio**
sample 1	0.0	0.0	13.4	39.3	32.0
sample 2	0.0	0.0	0.0	32.2	23.1
sample 3	0.0	8.6	36.7	99.4	4.3
sample 4	0.0	0.0	52.3	83.0	36.1
sample 5	0.0	5.8	42.5	7.6	23.0
Means	0.0	2.9±1.81*	29.0±9.67*	52.3±16.93*	23.7±10.95*

The ratio of the area due to the PI-I probe over the area due to the 18S rRNA probe

* Standard Error (SE) = SD/√n

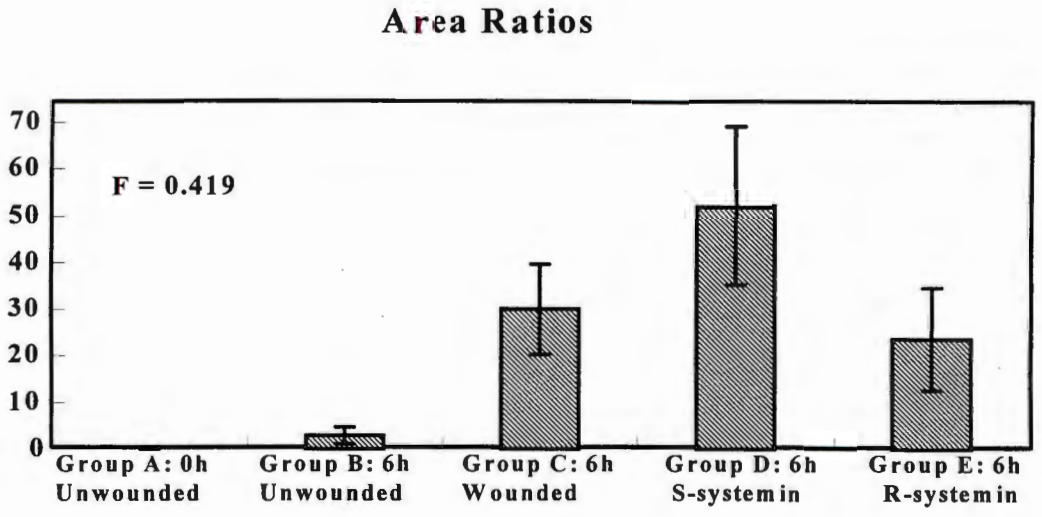


Fig. 6.6 Results of the systemin induction experiment. The data are shown in Table 6.2.

To examine the significance of the change in PI-I mRNA level between the negative control group (B) and the experimental groups (C-E), a two-sample analysis was applied [Manual E]. Each group's mean was compared with other group. A significant difference was found between the mRNA level of group B and those of the last three groups (C-E). However, no significant differences in mRNA levels were found between any of the groups C-E (wounded 6h, s-systemin 6h, r-systemin 6h).

6.4 Production of polyclonal antisera against MBP-systemin fusion protein

6.4.1 Production of polyclonal antibodies

The fusion protein was denatured with guanidine hydrochloride (see Chapter 4) shortly before injection into a rabbit in order to prevent renaturation. This process increased the possibility of r-systemin being exposed on the surface of the fusion protein, increasing its immunogenicity.

1 mg of the denatured protein was redissolved in 1 ml of phosphate buffer saline. This protein solution was injected into the rabbit once a week over a four-week period. In order to boost the rabbit immune response, it was injected again once in week 11 and 12. The antiserum was collected in week 8 and 13 (Prof. von Wechmar, personal communication). The time table is shown in Table 6.3.

Table 6.3 Time table for antigen injection (inj) and antiserum collection (col).

Week	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Inj	Inj	Inj	Inj				Col			Inj	Inj		Col

6.4.2 Western blotting

The protein samples were separated on a 13.5% SDS polyacrylamide gel. The undigested MBP fusion protein, 72 h digested MBP fusion mixture, synthetic systemin peptide, and the mixture of low m.w. marker and high m.w. marker were run on the same gel.

The separated proteins in the gel were transferred to a membrane to detect the binding efficiency of the polyclonal antibodies. The membrane was incubated with 5000 fold diluted antiserum (collected in week 8) for antigen-antibody conjugation. Excess antibodies were washed off from the membrane. Then the membrane was immersed in a biotin-labelled goat-anti-rabbit IgG for a secondary binding. The biotin on the secondary antibody was then bound to peroxidase-labelled streptavidin. Horseradish peroxidase (HPO) catalyses the oxidation of diacylhydrazides like luminol in the presence of hydrogen peroxide (H_2O_2). A reaction product in an excited state is thus formed [60, 69], which decays to the ground state by emitting light. Strong enhancement of the light emission is achieved by 4-iodophenol, which acts as a radical transmitter between the formed oxygen radical and luminol. The membrane was wrapped in clingfilm and exposed to an X-ray film.

After the binding experiment, the membrane was stained with a coomassie blue staining solution to determine the quantity of loaded protein samples and their sizes (Fig 6.7).

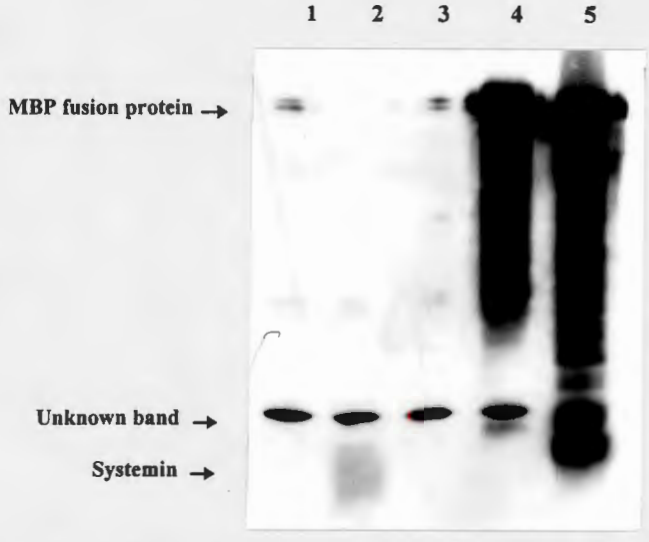


Fig. 6.7 Western blotting result. Lanes: 1. protein m.w. marker; 2. 5 μg of s-systemin; 3. 0.5 μg of r-systemin; 4. undigested MBP-fusion protein; 5. digested MBP-fusion protein.

6.5 Discussion and summary

6.5.1 Synthesis of the PI-I mRNA probe

In order to quantify the PI-I mRNA, a probe was made. A part pf the PI-I gene was amplified by PCR. The PCR product showed a size of about 370 bp on gel. This DNA fragment which was cloned into a pBluescript plasmid was sequenced. The sequence matched the known PI-I sequence. The insert in the plasmid was labelled with DIG-dUTP using a PCR method, and the PCR product was used as a probe in northern blotting experiments.

The PCR product contained only an average of one digoxigenin molecule per 20-25 nucleotides because the *Taq* polymerase does not recognise DIG-dUTP as efficiently as it recognises dTTP [Manual B]. The overall yield of DIG-labelled PCR-product synthesised was lower than the yield of a comparable unlabelled product. The reasons for this decrease in yield are unknown [Manual B].

6.5.2 Test for biological activity of r-systemin

In a pilot experiment, the PI-I mRNA level in the "unwounded 6h" group increased slightly but not as much as in the "wounded 6h" group. The same procedure was used in the systemin induction experiment. The production of proteinase inhibitor I mRNA in the young tomato plants increased significantly in the three experimental groups "wounded 6h", "s-systemin 6h", and "r-systemin 6h" (groups C-E). The PI-I mRNA level in the group "unwounded 6h" (B) increased only slightly. This evidence suggested that r-systemin triggers the accumulation of PI-I mRNA in the same manner as s-systemin or wounding in tomato plants. We suggest that r-systemin induces the same biological responses as s-systemin as well as native systemin.

It is important to use healthy plants in the tests because plants suffering from environmental stress produce an unstable expression level of PI-I mRNA. For example, the leaves of the tomato plants which were grown indoors turned yellow due to insufficient light intensity and poor light quality. When these plants were used in experiments, some unwounded plants were found to have a high PI-I expression and some wounded plants had no PI-I mRNA. This problem was circumvented by growing the plants in a greenhouse.

6.5.3 Western blotting

Polyclonal antibodies against the MBP-systemin fusion protein were raised. They bound to both, the r-systemin and the s-systemin in a western blotting experiment, but the binding was different. The s-systemin band (Fig 6.7, lane2) was fuzzy and diffuse unlike the band of the r-systemin released from fusion protein (Fig 6.7, lane 5) which formed a sharp band. The protein samples were dialysed against the same buffer before electrophoresis or a higher percentage gel was run. The results were reproducible. The reasons for this binding difference are unknown.

One extra band appeared in every lane (even in the molecule weight marker lane) on the X-ray film. It might due to a tank buffer contamination, as it was not seen in the other western blotting tests.

More experiments are needed to examine the specificity of the polyclonal antibodies, especially when they are applied to a total tomato protein extract. If the antibodies are shown to bind only to systemin or its precursor (pro-systemin) but not to other plant proteins, this antiserum could be used to detect the change of the systemin peptide level in the tomato plants under different stress conditions. It is potentially a valuable tool for any further research on systemin and its functions.

Chapter 7 Conclusions

The peptide sequence of systemin is known [55]. In order to understand its biological function in tomato plants, we compared the systemin peptide sequence with other plant protein sequences using a computer programme called GCG [Manual E]. Many plant cell wall proteins such as the carrot 33-kDa protein and soy bean 1A10 protein, were found to be proline rich and to have a ProProLys motif like systemin does. The biological functions of these cell wall proteins are still not very clear. It was suggested that most of them may play a role in the plant defence mechanism. More research is needed to decide whether systemin is a member of this proline-rich cell wall protein family.

We created a 3 D tertiary structure model of systemin based on its amino acid sequence. The picture is shown in Chapter 2. Dissolved in organic solvent for MNR analysis, systemin was shown to lack a persistent secondary structure [61]. A left-handed, 3_1, poly (L-proline) II (PPII) secondary structure was proposed to be the main structural feature in systemin. It is possible that a stable secondary structure can form after systemin peptide binds to either other binding factors or to a membrane receptor. A 50 kDa protein on the tomato cell membrane which binds to systemin has been isolated [70].

Due to the low expression level of native systemin in tomato leaves, purification is difficult. In order to obtain large amounts of the peptide, we decided to express systemin in a bacterial expression system. A single strand DNA sequence encoding the systemin peptide was designed based on *E. coli* codon preference and synthesised on an oligonucleotide synthesiser. The single-stranded oligonucleotide was amplified using PCR to produce double-stranded DNA for cloning. We cloned the synthetic gene into a small, high-copy number plasmid, pUC18. The DNA sequence of the insert was determined and confirmed before the gene was subcloned into a pMAL-pk plasmid for expression in *E. coli* cells.

In the pMAL-pk system, systemin was translated together with a maltose binding protein (MBP) as a fusion protein and subsequently purified on an amylose resin column. When total *E. coli* periplasmic space proteins were loaded onto the column, the MBP bound strongly to maltose on the resin while other proteins were washed off. The fusion protein was later eluted with a maltose buffer. The eluted fusion protein was redissolved in digestion buffer for the enterokinase digestion reaction. The recombinant systemin was thus released from the fusion protein.

The digestion mixture was loaded onto a reverse-phase HPLC C18 column. From the HPLC profile, we could determine the digestion efficiency. HPLC is the method of choice to purify the small peptide and/or separate it from the undigested fusion proteins and MBP. The physical characteristics and biological function of this peptide could then be tested.

The physical characteristics of a native systemin were described by Pearce [55]. He showed that synthetic systemin (s-systemin) has the same retention time as native systemin. It was also shown to induce the accumulation of proteinase inhibitors in the tomato plants [55, 56]. Therefore, s-systemin was employed in our experiments as a positive control to compare with our recombinant systemin (r-systemin).

The physical characteristics of r-systemin were identical to those obtained for the s-systemin. Their amino acid composition, amino acid sequence, and retention time on the HPLC have been compared. The molecular weight of r-systemin analysed on a mass spectrometer was identical with the one deduced from the known amino acid composition.

The biological function of r-systemin was examined. The PI-I mRNA level appeared to be very low in the control group, in which the tomato plants were unwounded. In comparison, the group of plants which had been incubated in buffer containing r-systemin, had a much higher PI-I mRNA level. The PI-I mRNA levels in plants which were either supplied with s-systemin or mechanically wounded were also high. These results suggest that r-systemin

triggers the accumulation of PI-I mRNA, which is one of the well known wounding responses.

To characterise r-systemin inside the cells further, polyclonal antibodies against systemin-MBP fusion protein were raised in a rabbit. In a pilot western blotting experiment, antibodies bound to r-systemin and s-systemin. These antibodies should be explored further to find out whether they bind only to native systemin or pro-systemin polypeptide in a western blotting experiment which uses total tomato proteins. They can possibly be applied in studies quantifying the systemin level change in tomato plants.

In our experience the following steps appeared to be the most difficult ones:

1. To release the small peptide from the fusion protein.

   We found that the fusion protein could not be digested unless it was incubated at high temperature for more than 48 hours. The concentration of the protease (enterokinase) was also crucial. If the concentration was too high, non-specific digestion occurred. We concluded that a long incubation time, high digestion temperature and a proper enzyme/fusion protein ratio were important to release the systemin from the fusion protein specifically and efficiently.

2. To detect the intact small peptide.

   Normally a gel electrophoresis system using high percentage polyacrylamide is chosen to analyse small peptides, but this system also has disadvantages. Sensitivity is lost for small peptides because they diffuse easier from the gel than bigger peptides and proteins during staining and destaining procedures, so that it is difficult to visualise them. We used reverse phase HPLC as an alternative approach. This system is very sensitive for the identification of low molecular weight peptides even at low concentrations.

3. To test the biological function of the recombinant peptide.

   We had problems with a large background of non-specific reactivity in the biological function tests initially. The background was due to poor handling techniques and/or possibly because stressed plants were used in the experiments. Special care must be taken to protect the plants from insects attack before or during the experiments and to prevent the accumulation of PI-I triggered by other factors.

4. To obtain the materials.

   The yield of the r-systemin product was low. Losses certainly occurred during the multi-step purification. An expression system with high yield or with less purification steps could be used.

In summary, a bacterial expression system was chosen to express the small plant peptide, systemin, *in vitro*. Recombinant systemin was purified from its host (*E. coli* cells) successfully, and its physical characteristics and biological function were identical to synthetic systemin.

Chapter 8 Materials and methods

8.0 Contents

8.1 DNA techniques

8.1.1 DNA isolation and plasmid preparations

8.1.1.1 Small scale plasmid preparation

This method was adapted from an alkaline lysis large-scale preparation method [1].

1. A single colony of the plasmid containing culture was grown overnight in 3 ml of Luria broth (LB; see Appendix) in a sterile test tube with a selective antibiotic added.
2. To collect the bacterial cells, 1 ml of the overnight culture medium was centrifuged in a microfuge tube at 12,000×g for 5 minutes at room temperature.
3. The cells were resuspended in 200 µl of glucose P1 buffer (Appendix). The tube was incubated on ice for 5 minutes.
4. 200 µl of P2 buffer (Appendix) was added and incubated on ice for 5 minutes.
5. 200 µl of P3 buffer (Appendix) was added, mixed gently and incubated for 5 minutes on ice.
6. The cell debris was spun down at 4°C for 10 minutes. The supernatant was transferred to a clean microfuge tube.
7. 1 µl of RNase (20 mg/ml) was added to the supernatant to digest the RNA at 37°C for 30 minutes.
8. 1 µl of Proteinase K (10 mg/ml) was added to above solution and incubated at 37°C for 30 minutes.
9. 300 µl of equilibrated phenol (Appendix) and 300 µl of chloroform were added to the supernatant. The sample was vortexed thoroughly and centrifuged for 10 minutes at 4°C. The upper aqueous phase was carefully collected and transferred into a new tube. 600 µl of chloroform was then added to the tube, mixed well and centrifuged again. The upper aqueous phase was collected.
10. An equal volume of isopropanol and 1/10 volume of 3 M NaOAc buffer, pH 5.2 were added to the supernatant to precipitate the plasmid DNA at –20 °C overnight.

11. The DNA was collected by centrifugation at 12,000×g for 20 minutes at 4 °C.
12. The DNA pellet was washed twice with 70% ethanol to remove salt. The pellet was dried briefly and redissovled in 10 µl H_2O.

8.1.1.2 Large scale plasmid preparation

The method for large scale plasmid DNA preparation consists of two parts. Part I, involves alkaline lysis, which separates chromosomal DNA from plasmid DNA. Part II, requires centrifugation in CsCl/ethidium bromide density gradients. This step allows a second separation of plasmid DNA from protein, chromosomal DNA and RNA. Following ultracentrifugation, a single band containing supercoiled plasmid is extracted with a needle and a syringe from the centrifuge tube [1].

Part I: Alkaline lysis

1. A single colony of the required plasmid containing the desired insert was grown overnight in 10 ml of LB with a selective antibiotic in a sterile McCartney bottle at 37°C.
2. A 1 litre flask, containing 250 ml LB with a selective agent, was inoculated with 1 ml of the overnight culture. This culture was grown at 37°C overnight with vigorous shaking, until the optical density at 600 nm (OD_{600}) reached 4.
3. The cells were collected by centrifugation at 6,000×g for 10 minutes at 4°C and resuspended in 10 ml P1 buffer. The centrifuge tube was incubated on ice for 5 minutes.
4. 10 ml P2 buffer was added gently, mixed well and allowed to stand on ice for 5 minutes.
5. 10 ml P3 buffer was added and the tube was incubated on ice for another 5 minutes.
6. The debris was pelleted by centrifugation at 27,000×g for 10 minutes at 4°C.
7. The supernatant was transferred to a new centrifuge tube. An equal volume of isopropanol was added to precipitate the DNA for 30 minutes at –70°C.

8. The nucleic acid pellet was collected by centrifugation at 48,000×g for 20 minutes at 4°C. The pellet was briefly dried and then redissolved in 2 ml sterile H_2O. 10 µl of RNase (20 mg/ml) was added to the DNA and incubated at 37°C for 1 hour. 10 µl of Proteinase K (10 mg/ml) was then added and incubated at 37°C for 1 hour.

Part II: CsCl/EtBr equilibrium centrifugation

9. 4.29 g CsCl and 0.202 ml EtBr (10 mg/ml) were added to the enzyme/plasmid solution. The final volume was made up to 5.5 ml with TE buffer (Appendix).
10. The solution was transferred to a 5 ml ultracentrifuge tube and centrifuged at 289,000×g (50,000 rpm in a Beckman Vti 65.2 rotor) for 16 hours at 20°C.
11. The plasmid DNA band was extracted with a syringe and needle.
12. An equal volume of isoamylalcohol was added to extract the EtBr for a few times until no red fluorescence was visible in the solution.
13. The plasmid DNA was dialysed against 2 litres of cold 10 × diluted TE buffer for four hours at 4°C, and the buffer was changed once.
14. The plasmid DNA was precipitated with 2 volumes of 100% ethanol and 1/10 volume of 3 M sodium acetate at –20°C overnight.
15. The DNA was pelleted by centrifugation. The pellet was washed twice with 70% ethanol and air dried. It was dissolved in 100 µl TE buffer.

8.1.1.3 Small scale plant genomic DNA preparation

The "Supaquick" DNA extraction method is a simple and quick way to extract plant genomic DNA.

1. Two leaf disks were collected in a microfuge tube.
2. A pinch of carborundum was added for grinding the leaves to pulp with a clean glass rod.
3. 400 µl of "supaquick" buffer (Appendix) was added and mixed well. The tubes were incubated at 60°C for 10 minutes.

4. An equal volume of chloroform/isoamylalcohol (24:1) was added and mixed well.
5. The tubes were centrifuged at 11,000×g for 10 minutes. The upper aqueous phase was transferred to a clean microfuge tube.
6. 0.4 volume of 0.25 M NaCl and 3 volumes of cold 100% ethanol were added, mixed gently and chilled at –20°C for 30 minutes.
7. The tubes were centrifuged at 11,000×g for 10 minutes at 4°C, and the supernatant was decanted. The pellet was washed twice with 70% ethanol and dried briefly. The pellet was redissolved in 100 µl TE buffer.

8.1.2 Enzyme digestion

8.1.2.1 Restriction enzyme digestion

The restriction enzyme (R.E.) digestion is often used in DNA techniques. The 10 × enzyme buffer is normally supplied with the particular restriction enzyme. Different R.E. sometimes must be digested in different environment. If two enzymes require two different buffers for a double digestion, a phenol/chloroform extraction and an ethanol precipitation is applied to the DNA between the digestions to change the buffer.

1. 10 × buffer, H_2O and sufficient restriction enzyme(s) were added to double-stranded DNA.
2. The tube was incubated at the suggested digestion temperature for 1 hour.
3. 1 µl of 0.5 M EDTA was added to stop the reaction.

8.1.2.2 Nuclease PI digestion

Nuclease PI cleaves 3' phosphoester bonds [10]. It was used to delete the 3' overhanging bases in order to create blunt-ended DNA fragments for cloning. PI nuclease powder (Boehringer Mannheim) was dissolved in 0.03 M NaOAc, pH 5.2 and stored at –20°C. The digestion time and enzyme/DNA ratio was controlled to prevent over-digestion. It was found

that a 0.025 unit of PI nuclease could digest 0.5 pmol of "sticky end". Restriction enzyme buffer H (Appendix) was used for the nuclease PI digestion. The reaction was incubated at 37°C for 15 minutes.

8.1.3 Electrophoresis

8.1.3.1 Agarose gel

Agarose gel electrophoresis is a simple and highly effective method for separating and identifying 0.5 to 25 kb DNA fragments [1]. The agarose concentration in the gel depends on the size of the DNA fragments to be separated. Two electrophoresis buffers are commonly used, 50 × TAE buffer (Appendix) and 10 × TBE buffer (Appendix).

1. The desired amount of electrophoresis-grade agarose was added to a volume of electrophoresis buffer sufficient for constructing the gel. EtBr was added to the gel solution and tank buffer to a final concentration of 1 μg/ml; it helped to trace the DNA fragments during the run.
2. Melted agarose was poured into a sealed gel casting platform and the gel comb was inserted.
3. The comb was removed from the hardened gel. The gel was placed in an electrophoresis tank which was filled with electrophoresis tank buffer. Loading buffer (Appendix) containing tracing dyes was mixed with the sample and the sample was loaded into the well.
4. The gel was run at 60 to 100 V until the tracing dye migrated to the end of the gel.

8.1.3.2 Non-denaturing acrylamide gel

Non-denaturing acrylamide gels provide better resolution than agarose gels especially for small DNA fragments. The percentage of acrylamide depends on the size of DNA fragments.

1. The glass plates with spacers were assembled to cast the gel.

2. The gel solution consisted of 10 × TBE buffer, 29:1 acrylamide/bisacrylamide solution, and distilled water.
3. 25 µl TEMED and 250 µl of 10% ammonium persulfate were added to every 50 ml gel solution. The mixed gel solution was poured between the plates, the gel comb was inserted, and the gel was left to set.
4. The comb was removed from the set gel. The gel tank was filled with 1 × TBE buffer. Plates were attached to gel electrophoresis apparatus.
5. The samples were mixed with loading buffer and loaded onto the gel with a Hamilton syringe.
6. The gel was run at about 100 to 150 V until sufficient resolution had been obtained.
7. The gel was stained for 5 to 10 minutes in 0.5 µg/ml EtBr to visualise DNA.

8.1.3.3 Denaturing polyacrylamide gel

Denaturing polyacrylamide gels can resolve oligonucleotides from 2 to 300 bases, depending on the percentage of polyacrylamide used [1].

1. The gel casting apparatus was assembled.
2. The gel solution containing 7M urea was prepared (Appendix) and filtered with a 0.45 µm filter.
3. 100 µl of 10% ammonium persulfate and 15 µl TEMED were added to every 30 ml gel solution, mixed well and poured into the space between the plates. The comb was then inserted and the gel was allowed to set.
4. After the polymerization of the gel was completed, the comb was removed and the plates were attached to the gel electrophoresis tank which was filled with 1 × TBE buffer.
5. The gel was preheated by running the gel at 250 to 300 V for 20 minutes.
6. An equal volume of formamide was added to the sample solution. The samples were incubated at 50°C for 5 minutes to denature the secondary structure of

oligonucleotides. After 5 minutes, the tubes were chilled on ice immediately to prevent renaturation.

7. The samples were loaded onto the gel with a Hamilton syringe. Some loading buffer containing tracing dyes was loaded into an empty well.

9. The gel was run at 250 to 300 V in order to keep the denaturing state of oligonucleotides at a high temperature (about 42°C) which would assure good resolution.

10. After switching off the voltage the gel was removed from plates, and stained with 0.5 μg/ml EtBr for 5 to 10 minutes.

8.1.3.4 Sequencing gel

A sequencing gel is a high-resolution denaturing polyacrylamide gel (usually 5-6%) designed to fractionate radiolabelled ssDNA on the basis of size. Urea (7 M) in the sequencing gels reduces the occurrence of DNA secondary structure. A thin gel (0.1 mm) is normally run at high voltage and high temperature in order to achieve a better resolution of the DNA bands on a X-ray film [1].

1. The gel plates were cleaned thoroughly. The spacers were wetted and laid on the three edges of the plates. All three sides were clamped.

2. 4.7 g acrylamide, 0.25 g bisacrylamide, 48 g urea, 10 ml 10 × TBE, and 40 ml distilled H_2O were mixed for a 5% acrylamide gel solution.

3. 50 ml gel mix was filtered through 0.8 μm filter into a beaker. Then 45 μl 50% fresh ammonium persulfate and 45 μl TEMED were added and mixed well.

4. The gel solution was drawn into two 25 ml syringes.

5. The gel solution was very smoothly injected from one side of the plate set, so avoiding the formation of any air bubbles.

6. The comb was inserted and the top side was clamped.

7. The samples were denatured before loading by heating at 95°C for 5 minutes. The gel was run at 90 W for 1 hour or longer at 40-42°C.

8. The gel was laid on a piece of Whatman 3MM paper and covered with clingfilm, it was dried at 70°C for 1 hour under vacuum.

8.1.4 Cloning methods

8.1.4.1 Phosphatase digestion

Calf intestinal alkaline phosphatase (CIP) removes 5'-phosphate groups from linear DNA.

1. Closed circular plasmid DNA was digested with suitable restriction enzyme(s) and the extent of digestion was analysed by electrophoresis through an agarose gel. After the digestion was complete, the sample was extracted with phenol/chloroform and the DNA was precipitated with ethanol.
2. The pellet was dried briefly and redissolved in 1 × CIP dephosphorylation buffer (Appendix). Sufficient CIP for dephosphorylation was added to the sample and incubated under appropriate conditions. The amount of CIP added, and the incubation conditions varied depending on the type of plasmid termini (protruding or blunt termini).
3. The DNA was phenol/chloroform extracted and ethanol precipitated after dephosphorylation.

8.1.4.2 Ligation

To assay whether dephosphorylation has been successful, a series of test ligations and transformations were set up. Ligation of blunt ended DNA is a comparatively inefficient reaction, 10 to 100 times more enzyme is recommended for blunt-end ligation than cohesive-end ligation to achieve an equal efficiency [46]. Two different concentrations of T4 DNA ligase were available. 5 units/μl ligase (high concentration) was used for blunt-end ligation and 1 unit/μl ligase (low concentration) was used for sticky end ligation.

The molar ratio of insert to plasmid of 2:1 was chosen for the ligation reactions. 10 × ligation buffer (Appendix), H_2O and sufficient T4 ligase were mixed in the tubes. The samples were incubated in an ice water bath overnight.

8.1.4.3 Competent cells preparation

We used the method of Chung to prepare competent bacterial cells [16].

1. Bacterial cells (*E. coli*) were grown up to the early log phase (OD_{600} = 0.3-0.6) in LB medium.
2. The cells were pelleted by centrifugation at 12,000×g for 10 minutes at 4°C, and resuspended in 1/10 volume of transformation buffer (TSB buffer, Appendix).
3. The cell suspension was incubated on ice for 10 minutes. The cells were then aliquoted into small microfuge tubes and stored at −70°C.

8.1.4.4 Transformation

The method used for transformation [16] involved the following steps:

1. One third of the sample containing the ligation mixture was added to 50 µl competent cell suspension. The cell sample stood on ice for 30 minutes.
2. 450 µl of TSB glucose buffer (Appendix) was then added to the tubes and mixed well. The cell/plasmid mix was incubated at 37°C with shaking (200 rpm) for 1 hour.
3. 200 µl of the cell culture was plated on antibiotic-containing agar plates for selection of transformants. The plates were incubated at 37°C overnight.
4. The colony number on each plate was recorded.

8.1.5 Probe labelling

8.1.5.1 Radioactivity end-labelling

To end label DNA and RNA molecules, such as oligonucleotides, restriction fragments, and mRNA with [γ^{32}P] dATP, the strands must have a 5'-OH group at the ends. The labelled oligonucleotides can be used either as a hybridisation probe or as a sequencing primer.

1. Preparation of a 1 ml syringe column for purification: a plug of silanised glass wool was placed at the end of the syringe. G-25 or G-50 Sephadex (medium) suspended in STE buffer was poured into the syringe.
2. 50 µl of STE buffer (Appendix) was loaded onto the column and centrifuged at 800 rpm for 3 minutes. This step was repeated twice.
3. The labelling reaction included 50 pmol oligonucleotides, 10 × PNK buffer (Appendix), [γ^{32}P] dATP, T4 polynucleotide kinase (10-15 units) and H_2O in a final volume of 50 µl. The solution was incubated at 37°C for 1 hour.
4. The column was rinsed in the same manner as step 2 before loading the sample from step 3.
5. A microfuge tube was placed in a centrifuge tube to collect the reaction solution which had passed through the column.
6. The reaction was stopped by adding 1 µl 0.5 M EDTA.
7. The reaction mixture was loaded onto the column and centrifuged at the 800 rpm for 3 minutes. The labelled DNA was transferred to a new clean tube and stored at –20°C.

8.1.5.2 DIG end-labelling

The DIG 3' end labelled DNA or oligonucleotides can be used as hybridisation probes. The labelling kit was supplied by Boehringer Mannheim. A poly-A tail with about 1/10 DIG-dUTP was generated at the 3' end of oligonucleotides during the labelling reaction.

1. Reagents were added to a microfuge tube in the following order: 5 × reaction buffer (Appendix), $CoCl_2$ solution, 1 mM DIG-11-dUTP, 100 pmol oligonucleotide, 10 mM dATP, terminal transferase and H_2O.
2. The reaction was incubated at 37°C for 1 hour and then placed on ice.
3. 1 µl glycogen solution and 1 µl 0.2 M EDTA, pH 8.0 were added to stop the reaction.
4. The labelled oligonucleotide was precipitated by mixing it thoroughly with 0.1 volume of 4 M LiCl and 2.5-3.0 volumes of pre-chilled 100% ethanol, and incubated at –70°C for 30 minutes.
5. The labelled DNA was collected by centrifugation at 12,000×g for 15 minutes at 4°C. The pellet was washed twice with 70% ethanol, then dried briefly and resuspended in 20 µl sterile H_2O, stored at –20°C.

8.1.6 PCR

8.1.6.1 Oligonucleotide PCR

This protocol was used to convert the single-stranded oligonucleotide to double-stranded DNA with two primers [50].

1. PCR amplification solution contained 10 × PCR buffer (Appendix), 1/10 volume of 2 mM dNTPs, 200 pmol of each primer, 1 µg oligonucleotide template, 5 units of Vent DNA polymerase and H_2O. 30 µl of sterile liquid paraffin was added to prevent evaporation at high temperature.
2. The PCR cycle was: 92°C for 20 seconds, 65°C for 30 seconds, and 72°C for 60 seconds. This PCR cycle was repeated 35 times.

8.1.6.2 Genomic DNA PCR

When genomic DNA is used as a PCR template, the PCR begins with a high temperature step (92-94°C) to denature the double-stranded DNA.

1. 10 × PCR buffer, dNTPs (0.2 mM), 20 pmol of each primer, 0.5 unit DNA polymerase (DynaZyme), 1 μg total tomato genomic DNA and H_2O were mixed in a microfuge tube. The solution was overlaid with liquid paraffin.
2. The sample was incubated at 94°C for 90 seconds to start the amplification. The following cycles were repeated 30 times: 94°C for 20 seconds; 57°C for 30 seconds; and 72°C for 45 seconds. Finally, the tubes were kept at 72°C for further 180 seconds.

8.1.6.3 PCR screening

In our hands the screening method described below proved to be the quickest way to examine recombinant plasmids. The multicloning site in the plasmid is amplified using PCR methodology. The sizes of PCR products, are expected to be bigger in recombinant plasmids as compared to non-recombinant plasmids because of an insertion in the multicloning site [79]. The PCR products are examined on either a polyacrylamide gel or an agarose gel (Chapter 8, §1.3).

1. The colony was grown overnight in 3 ml of liquid LB medium containing a selective agent, e.g. ampicillin, overnight.
2. 5 μl of overnight culture was transferred to a microfuge tube and 95 μl sterile H_2O was added. The plasmid was released from the cells by boiling in a water bath for 3 minutes.
3. A solution including 10 × PCR buffer, dNTPs (0.2 mM), 20 pmol of each primer, $MgCl_2$ (3 mM), 0.5% Tween-20 and H_2O was pre-mixed for PCR. 25 μl of boiled bacterial solution and 25 μl of above PCR solution were added into a new PCR microfuge tube. 1 unit of *Taq* polymerase was added to the mixture and the solution was overlaid with 50 μl of liquid paraffin.
4. The following PCR amplification cycle was repeated 30 times: 92°C for 10 seconds; 50°C for 30 seconds; 72°C for 60 seconds. Full extension was carried out at 72°C for 300 seconds after the last cycle.

8.1.6.4 PCR DIG-labelling

This method incorporates DIG-dUTP into a double-stranded PCR product. In the dNTPs solution, DIG-dUTP is mixed with dTTP in a 1:2 ratio [Manual B]. A control sample is amplified with non-DIG dNTPs mixture at the same time.

1. 10 × PCR buffer, 0.2 mM of non-DIG dNTPs mixture or DIG dNTPs mixture, 15 pmol of each primer, 5 ng of plasmid DNA with insert, 1 unit of *Taq* DNA polymerase, and H_2O were mixed, the solution was covered with 30 µl of liquid paraffin.
2. The DNA was amplified using the following cycle conditions: 94°C for 20 seconds, 57°C for 30 seconds, 72°C for 45 seconds, repeated 30 times. To fully extend the PCR product, the solution was maintained at 72°C for a further 180 seconds.

8.1.7 Recombinant DNA screening method

8.1.7.1 Southern blotting

Southern blotting is the transfer of DNA fragments from an electrophoresis gel onto a membrane support. The membrane carries a reproduction of the banding pattern of the gel. After immobilization of the DNA fragments, the DNA can be subjected to hybridisation analysis, enabling bands with sequence complementarity to a labelled probe to be identified.

Southern blotting occurs via upward capillary transfer of DNA, using a high-salt transfer buffer to promote binding of DNA to the nitrocellulose or nylon membrane. Permanent immobilisation is achieved by UV irradiation (nylon) or baking at 80°C (nitrocellulose)[1].

1. The DNA was electrophoresesed on an agarose gel. The gel was stained and photographed. The distances between the bands and the wells were measured.
2. The gel was rinsed in distilled H_2O and then placed in a container with 1.5 M NaCl,

and 0.5 N NaOH to denature the DNA by shaking slowly at room temperature for 45 minutes.

3. The gel was rinsed with distilled H_2O, then transferred into a tank containing neutralisation solution (1 M Tris, pH 7.4, 1.5 M NaCl) and then gently shaken for 30 minutes. The neutralisation solution was changed once and the gel was incubated for 15 minutes.
4. The rinsed gel was transferred to the platform for capillary blotting. The tank was filled with 20 × SSC. The DNA was blotted onto a membrane at room temperature overnight.
5. The membrane was air dried. The DNA was fixed onto the filter by either exposing to UV light in a UV-crosslinking oven or incubating in an oven (80°C) for 1 hour.

8.1.7.2 Colony blotting

This method, although time consuming, is the most economical way to screen a large number of colonies for recombinant plasmid.

1. Cells from a single colony were inoculated with tooth picks twice from the overnight cultures onto two plates. The two plates, both contained LB and agar with a selective agent. However, one had either a nylon or a nitrocellulose membrane placed on the agar surface. Both plates were incubated at 37°C overnight.
2. After overnight incubation, the plate without membrane was stored at 4°C.
3. The membrane was peeled from the plate using blunt-ended forceps and place colony side up, on the SDS-impregnated Whatman 3MM paper for 3 minutes.
4. The membrane was transferred to a second sheet of Whatman 3MM paper saturated with denaturing solution (5 minutes).
5. The membrane was transferred to the last sheet of Whatman 3MM paper, which had been saturated with 2 × SSC, for 5 minutes.
6. The membrane was air dried with the colony side up.
7. The DNA was fixed to the membrane by exposing to UV light or baking.

8.1.7.3 Hybridisation

In hybridisation, a single-stranded DNA or RNA molecule of defined sequence (the "probe") can anneal to a second DNA or RNA molecule that contains a complementary sequence (the "target"). The stability of the hybrid depends on the extent of base pairing that occurs. Experimentally, the analysis is usually carried out with a probe that has been labelled and target DNA that has been immobilised on a membrane support [46].

1. The membrane carrying the immobilised DNA was wetted in 5 × SSC (Appendix) in a hybridisation bag or tube.
2. About 1 ml of prehybridisation solution (Appendix) per 10 cm^2 of membrane was added into the bag or tube. The membrane was incubated at an optimised temperature for 1 hour.
3. If a double-stranded DNA was used as a probe, this dsDNA need to be heat denatured or alkali denatured shortly before transfer to the hybridisation solution (Appendix).
4. The prehybridisation solution was replaced with hybridisation solution.
5. The membrane was hybridised at a desired temperature with gentle shaking or rolling overnight.
6. The hybridisation solution was poured off. 2 × SSC/0.1% SDS was added into the bag or tube to wash the membrane for 10 minutes at room temperature and the washing solution was changed after 5 minutes.
7. For a more stringent washing procedure, a solution of 0.2 × SSC/0.1% SDS was used to wash the membrane at a desired washing temperature for 30 minutes, the solution being changed after 15 minutes.
8. The membrane was rinsed with 2 × SSC. If the probe was radiolabelled, the membrane was wrapped in plastic wrap and set up for autoradiography. If the probe was DIG labelled, a separate protocol was followed to inspect the hybridisation results (Chapter 8, §1.12).

8.1.8 DNA gel purification

The DNA samples were separated by electrophoresis on a non-denaturing acrylamide gel. The DNA bands of interest were then recovered by the following method [1].

1. The desired DNA band was cut out.
2. The gel slice was chopped into many fine pieces. If the gel was thin (<1 mm), this step could be omitted.
3. The gel pieces were transferred to a microfuge tube.
4. Sufficient elution buffer (Appendix) was added to cover the gel fragment with twice the volume of the gel. The tubes were incubated at 37°C overnight and rolled.
5. The supernatant solution was removed without polyacrylamide pieces.
6. Any residual DNA was recovered by rinsing the gel fragments with a small volume of elution buffer.
7. The DNA was precipitated with 2 volumes of 100% ethanol and chilled for 30 minutes at –20°C. The DNA was pelleted by centrifugation for 10 minutes at 12,000×g, the pellet was washed twice with 70% ethanol.
8. The air dried pellet was redissolved in TE buffer.

8.1.9 Primer design

To design a set of primers for sequencing or PCR, a programme from the GCG called "prime" was used [Manual E]. Certain factors are considered to be crucial for primer design [1, 20].

- The optimal length of the primers is considered to be about 20-30 nucleotides.
- The ratio of G,C and A,T content is about 1:1.
- The primer must be free of pronounced secondary structure.
- The annealing between primers should be minimised.
- The annealing temperatures of both primers should be close.
- No mismatch is tolerated at the 3' end of the primer.

8.1.10 Oligonucleotide synthesis

During synthesis on an oligonucleotide synthesiser, oligonucleotides are attached to a solid support [24]. The oligonucleotide product is then cleaved off from the resin and deprotected in 32% ammonia solution. The purity of the products is examined by gel electrophoresis.

8.1.11 Sequencing

A TaqTrack sequencing kit supplied by Promega was used. The kit is based on the dideoxy method (Sanger). This system allows for two different protocols. The end-labelled primer protocol, uses [γ^{32}P] dATP to label the sequencing primer, and the two step extension/labelling protocol is based on incorporating [α^{35}S] dATP into DNA by *Taq* polymerase.

If the DNA to be sequenced is double-stranded, the two strands should be denatured into single strands before starting a sequencing reactions by using an alkali denaturation method.

Protocol I: End-labelled primer sequencing.

Following this method, the primers were end-labelled with [γ^{32}P] dATP.

1. The radiolabelled primer was added and annealed to the denatured ssDNA template (1:1 molar ratio). Each set of four separate sequencing reactions (A, T, G, C), contained denatured DNA (1.6 pmol), *Taq* DNA polymerase 5 × buffer, labelled primer (2 pmol) and H_2O with a final volume of 25 µl, and incubated at 37°C for 10 minutes.
2. 1 µl of the appropriate d/ddNTP was added to each tube.
3. 4 units of sequencing grade *Taq* DNA polymerase were added to the annealed primer/template mixture and incubated at 37°C for 5 minutes.

4. 6 µl of the enzyme/primer/template mixture was transferred to each tube containing d/ddNTP (A, T, G, or C). The well mixed samples were incubated at 70°C for 10 minutes.
5. 4 µl of stop solution was added to each tube.
6. The solutions were heated at 90°C for 5 minutes before loading the sequencing gel.
7. The sequencing gel was dried and exposed to an X-ray film at –70°C until clear bands showed up.

Protocol II: Two step extension/labelling method

1. The double-stranded DNA was denatured.
2. The unlabelled primer was annealed to DNA in a microfuge tube with the following reagents: denatured DNA (1.6 pmol), primers (2 pmol), *Taq* DNA polymerase 5 × buffer, extension/labelling mixture and sterile H_2O (final volume 25 µl), and incubated at 37°C for 10 minutes.
3. 2 µl of [α^{35}S] dATP (approximately 10 µCi/µl) was added to the annealed primer/template mixture.
4. 4 units sequencing grade *Taq* DNA polymerase was added, the reaction was incubated at 37°C for 5 minutes.
5. For each set of sequencing reactions, 1 µl of the appropriate d/ddNTP mixture was added to each tube (A, T, G, or C).
6. 6 µl of extension/labelling mixture was added to each tube, incubated at 70°C for 5 minutes.
7. 4 µl of stop solution was added to each tube.
8. The final steps were the same as step 6 and 7 in protocol I.

8.1.12 Detection of DIG-labelled nucleic acids

With the DIG system, detection of DIG-labelled nucleotides can be accomplished by chemiluminescence or a colorimetric reaction. The detection kit was supplied by Boehringer Mannheim. We describe only the chemiluminescent procedure here. All incubations were performed at room temperature with agitation [Manual D].

1. After hybridisation and post-hybridisation washes, the membrane was equilibrated in buffer 1 (Appendix) for 1 minute.
2. The membrane was blocked with buffer 2-blocking reagent (Appendix) for 30 to 60 minutes.
3. The anti-digoxigenin-alkaline phosphatase (Fab) was 10,000 × diluted (75 mU/ml) in buffer 2, mixed gently.
4. Buffer 2 was decanted. The membrane was incubated for 30 minutes in the antibody solution (prepared in step 3).
5. The membrane was then washed twice in buffer 1 containing 0.3% Tween 20 (15 minutes per wash).
6. The membrane was equilibrated in buffer 3 (Appendix) for 2 minutes.
7. Lumigen^{TM} PPD [4-methoxy-4-(3-phosphate-phenyl)-spiro(1,2-dioxtane-3,2'-adam-antane) disodium salt] was diluted 100 times in buffer 3 (0.5 ml per 100 cm^2).
8. The membrane was incubated with diluted Lumigen^{TM} PPD for 5 minutes and wrapped in a plastic bag. Any bubble trapped in the bag was removed before sealing the bag.
9. The membrane was incubated at 37°C for 15 minutes to reach the steady-state. It was exposed to an X-ray film for a length of time (15 minutes to overnight, depending on the signal strength). The DNA-side faced the film.

8.2 Protein techniques

8.2.1 Protein isolation and purification

8.2.1.1 Total *E. coli* protein extraction

To extract the total proteins from *E. coli* cells, the procedure described in the "protein fusion and purification system manual" was followed.

1. 1 litre LB (with ampicillin) was inoculated with 10 ml overnight culture of cells containing the fusion plasmid.
2. The cells were allowed to grow 2 to 3 hours (OD_{600} = 0.4) before adding IPTG (Isopropylthiogalactoside; 0.3 mM). The cells were incubated at 37°C for another 2 hours.
3. The cells were harvested by centrifugation at 4,000×g for 20 minutes and resuspended in 50 ml lysis buffer (Appendix).
4. The sample was frozen at –20°C overnight and thawed in cold water the next day.
5. The cells were sonicated, the process was monitored by measuring the release of nucleic acid at OD_{260} until a maximum absorbance was reached (about 3 minutes).
6. NaCl was added to a concentration of 0.5 M. The solution was centrifuged at 9,000×g for 30 minutes.
7. The crude extract was diluted 5 times with column buffer (Appendix). This sample could be loaded onto the affinity column.

8.2.1.2 Periplasmic space protein extraction

1. 1 litre LB (with ampicillin) was inoculated with 10 ml overnight culture of cells containing the fusion plasmid.
2. The cells were grown 2 to 3 hours (OD_{600} = 0.4) before adding IPTG (0.3 mM). The cells were incubated at 37°C for 2 hours.

3. The cells were harvested by centrifugation at 4,000×g for 20 minutes. They were resuspended in 400 ml Tris·HCl, 20% sucrose, pH 8.0. EDTA (0.1 M) and incubated for 5-10 minutes at room temperature.
4. The solution was centrifuged at 8,000×g for 20 minutes at 4°C. The collected cells were resuspended in the same volume of ice-cold 5 mM $MgSO_4$ and stirred for 10 minutes at 4°C.
5. The supernatant was recovered by centrifugation at 8,000×g for 20 minutes at 4°C. This solution was the fluid resulting from the cold osmotic shock.
6. 4 ml of 1 M Tris·HCl, pH 7.4 was added to the cold osmotic shock fluid.
7. The fluid was dialysed against column buffer.

8.2.1.3. Affinity column purification of MBP fusion protein

The amylose resin was supplied by New England (BioLab). The resin is pre-swollen in 40% ethanol, its binding capacity is about 2-4 mg MBP/ml bed volume.

1. The resin was poured into a 2.5 × 10 cm syringe plugged with silanised glass wool. The column height to diameter ratio was less than or equal to 4.
2. The column was washed with 10 column volumes of column buffer (Appendix).
3. The extracted protein was loaded onto the column at a flow rate of about 1 ml/minute.
4. 8 column volumes of column buffer was loaded to wash off the unbound proteins.
5. The fusion protein was eluted with column buffer containing maltose (10 mM).
6. If desired, the column could be regenerated for re-use.

8.2.1.4 Reverse phase HPLC

A reverse phase C18 column (TSK-gel, ODS-80Tm, 4.4 mm ID × 15 cm, with a particle size of 5 μm, TosaHaas) was connected to HPLC (Millipore) to analyse the protease digestion results. For separation, a linear gradient from 100% solvent A (H_2O with 0.1% TFA) to 50% solvent B (60% acetonitrile with 0.1% TFA) over 60 or 120 minutes with a flow rate of 1 ml

per minute was set up, and the fractions were detected at 210 nm, the fractions of interest were collected manually.

8.2.2 Enterokinase digestion

Enterokinase was supplied by Biozyme (Great Britain). For some proteins, it is crucial to denature the proteins before digestion to increase the digestion efficiency.

1. The protein to be digested was redissolved in 10 mM Tris buffer, pH 8.0 to a final protein concentration of 2 mg/ml.
2. 100 units of enterokinase were added to every 10 pmol protein which had one digestion site per molecular. The sample was incubated at 42°C or at an optimised digestion temperature for 12-72 hours. The digestion efficiency was analysed by using electrophoresis or RP-HPLC.

8.2.3 Electrophoresis

8.2.3.1 SDS-PAGE

One dimensional gel electrophoresis under denaturing conditions (i.e., in the presence of 0.1% SDS) separates proteins based on molecular size as they move through a polyacrylamide gel matrix toward the anode [1]. For a monomeric protein, the detection of a single protein band after gel electrophoresis indicates the purity of that protein.

1. Two glass plates were assembled as a sandwich.
2. The separating gel solution was prepared by mixing 30% acrylamide/0.8% bisacrylamide (Appendix), 4 × Tris·HCl/SDS, pH 8.8 (Appendix), and H_2O. The solution was degassed for 10 to 15 minutes. Ammonium persulfate and TEMED were added.

3. The gel solution was poured into the space between the plates until a gap of 2-3 cm from the top edge of the gel remained. The surface was covered with a layer of isobutyl alcohol or H_2O.
4. The gel was allowed to polymerise.
5. The top layer of isobutyl alcohol or H_2O was removed completely before pouring in the stacking gel solution.
6. The stacking gel solution consisting of 30% acrylamide/0.8% bisacrylamide, 4 × Tris·HCl/SDS, pH 6.8, and H_2O was prepared. Ammonium persulfate and TEMED were added to the degassed solution.
7. The comb was inserted into the layer of stacking gel solution.
8. An aliquot of protein to be analysed was diluted with 1 volume of sample buffer (Appendix) and the samples were boiled for 5 minutes.
9. The gel comb was removed carefully from the set gel. The plates were attached to the gel tank. The tank was filled with 1× SDS/electrophoresis buffer (Appendix).
10. The samples were loaded with a Hamilton syringe into the wells.
11. The gel was run at 10 to 20 mA. After the Bromophenol Blue tracking dye reached the bottom of the separating gel, the power supply was disconnected.
12. The gel was carefully transferred to a container for staining.

8.2.3.2 Staining method

1. The gel was stained in a filtered coomassie brilliant blue R250 solution (Appendix) for 1 hour.
2. The gel was transferred to a destaining solution (Appendix). The destaining solution was changed a few times during destaining.
3. The destained gel was stored in storing solution (7% acetic acid).

8.2.4 Immunoblotting and immunodetection

Immunoblotting is used to identify specific antigens, recognised by polyclonal or monoclonal antibodies. Protein samples are solubilised, usually with SDS and reducing agents such as dithiothreitol (DTT) or 2-mercaptoethanol. Following solubilisation, the materials are separated by SDS-PAGE. The antigens are then electrophoretically transferred to a nitrocellulose, PVDF (polyvinylidene difluoride), or nylon membrane (Chapter 8, §2.4.1), a process that can be monitored by a reversible staining procedure [1].

The transferred proteins are bound to the surface of the membrane, providing access for immunodetection reagents. All remaining binding sites are blocked by immersing the membrane in a solution containing either a protein or detergent blocking agent (Chapter 8, §2.4.2). After probing with the primary antibody (Chapter 8, §2.4.3), the membrane is washed and the antibody-antigen complexes are identified with horseradish peroxidase (HPO) or alkaline phosphatase (AP) coupled to the secondary anti-IgG antibody (e.g., goat anti-rabbit IgG). The enzymes are attached directly or via an avidin (or streptavidin)-biotin bridge to the secondary antibody. Chromogenic or luminescent substrates are then used to visualise the binding results (Chapter 8, §2.4.4)[60].

8.2.4.1 Electrophoresis and electroblotting

The protein samples were separated by SDS-PAGE, and transferred to a PVDF membrane with a semidry blotting system.

1. When electrophoresis was complete, the gel sandwich was disassembled and the stacking gel removed.
2. Three sheets of Whatman 3MM paper saturated with transfer buffer (Appendix) were placed onto the anode plate.
3. An equilibrated transfer membrane was placed on top of Whatman 3MM paper stack. The gel was laid on top of the membrane.

4. 3 sheets of saturated Whatman 3MM paper were placed on top of the membrane. Air bubbles between each layer were avoided.
5. The top electrode was placed on the transfer stack.
6. High-voltage leads were carefully connected to the power supply. A constant current 200 mA was applied to transfer the proteins for about 1/2-1 hour.
7. The membrane was removed from the transfer stack and air dried. The gel was stained with coomassie blue staining solution after blotting.

8.2.4.2 Membrane blocking

A variety of agents are currently used to block binding sites on the membrane after blotting, e.g., Tween 20, PVP (polyvinyl pyrollidone), dry milk powder, casein, BSA and serum, we used PVP and a commercial blocking reagent from Boehringer Mannheim.

1. The PVDF membrane was wetted in methanol for a few seconds.
2. The membrane was transferred in H_2O for 1 minute.
3. The membrane was immersed in the blocking solution (Appendix) for 30 minutes and shaken at room temperature.
4. The membrane was washed twice with PBS (Appendix) containing 0.05% Tween 20 for 20 minutes at room temperature.

8.2.4.3 Immunoprobing

We used the rabbit polyclonal antibodies (against MBP-systemin fusion) for primary binding, and the biotinylated goat anti-rabbit IgG as the secondary antibodies. Subsequently, peroxidase-labelled streptavidin was added to bind to the biotinylated secondary antibodies. Finally, a chemiluminescent substrate (diacylhydrazides) reacting with peroxidase was applied to the membrane. All incubations were carried out at room temperature with gentle shaking.

1. The membrane was incubated in 5,000 × diluted rabbit serum in PBS for 2 hours.

2. The membrane was washed with PBS (plus 0.05% Tween 20) for 20 minutes.
3. 5,000 × diluted goat anti-rabbit IgG in PBS was used to incubate the membrane for 30 minutes.
4. The membrane was washed with PBS (Tween 20) for 20 minutes, buffer was changed once.
5. Streptavidin-POD was 10,000 × diluted in PBS. The membrane was placed in a tray with streptavidin and incubated for 30 minutes.
6. The membrane was washed in PBS (Tween 20) overnight. Washing solution was changed twice.

8.2.4.4 Visualisation with chemiluminesence

A BM chemiluminescence system (Boehringer Mannheim) was used for detection of the complex (Chapter 6.4.2).

After exposing the blot to the detection reagent, it must be processed quickly to avoid fading of the chemiluminescence reaction. This work must be performed in a dark room.

1. Excess buffer was drained from the washed blot and it was placed, protein side up, in a fresh container.
2. Premixed detection reagent (125 $\mu l/cm^2$) was added for a 1 minute incubation. Excess detection reagent was drained and the membrane was wrapped in gladwrap or a sealed plastic bag.
3. The membrane, protein side up was exposed to X-ray films for 1, 2, or 5 seconds.

8.3 RNA techniques

To obtain good preparations of eukaryotic mRNA, it is necessary to minimise the activity of RNases during cell lysis by using inhibitors of RNases. It is also important to avoid the accidental introduction of trace amounts of RNase from other potential sources in the laboratory [1].

8.3.1 Small scale total plant RNA isolation

We modified Verwoerd's method [77] to extract small amount of total plant RNA for northern blotting and hybridisation.

1. About 100 mg plant leaf tissue was collected and kept in a microfuge tube. Samples were frozen immediately in liquid nitrogen.
2. The frozen tissue was ground with a glass rod.
3. 0.5 ml of hot phenol extraction buffer (80°C, Appendix) was added to the frozen leaf powder, and shaken vigorously for 30 seconds.
4. 0.25 ml chloroform/isoamyl alcohol (24:1) was added and mixed well with the rest.
5. The tubes were centrifuged at 12,000×g for 5 minutes at 4°C. The upper aqueous phase was transferred carefully to a new microfuge tube.
6. an equal volume 4 M LiCl was added and mixed well, the RNA was precipitated by standing at –20°C overnight.
7. RNA was pelleted by centrifugation at 12,000×g for 15 minutes at 4°C.
8. The pellet was dissolved in 250 µl H_2O. RNA was washed with phenol/chloroform and precipitated again with 1 volume isopropanol and 1/10 volume 3 M NaOCl (pH 5.2) at –70°C for 1 hour.
10. The RNA was spun down at 12,000×g for 20 minutes. The pellet was twice washed with 70% ethanol.
11. The dried RNA was redissolved in DEPC H_2O (Appendix).

8.3.2 Electrophoresis

RNA is single-stranded, so most of them are able to form secondary structures by intramolecular base pairing and must therefore be electrophoresed under denaturing conditions if good separation is to be obtained. Denaturation is either achieved by adding formaldehyde to the gel and loading buffers or by treating the RNA with glyoxal and dimethyl sulfoxide (DMSO) prior to loading [1].

8.3.2.1 Denaturing agarose gel electrophoresis

We modified the glyoxal/DMSO denaturation method by using only glyoxal to denature the RNAs. The running buffer must be recirculated during electrophoresis to keep a constant pH throughout the tank buffer, because glyoxal dissociates from RNA at pH > 8.0.

1. An agarose gel with a desired percentage of agarose was prepared in 10 mM phospate buffer at pH 6.5. The melted gel solution was poured into a sealed gel plate stacks when the gel solution had cooled down to about 60°C.
2. The RNA samples were dissolved in 1 M glyoxal/10 mM phosphate buffer, pH6.5 buffer and denatured by heating at 50°C for 1 hour.
3. The samples were chilled on ice. 2 µl of loading buffer was added to every 10 µl of sample.
4. The sample was run at about 60 to 100 V with constant recirculation of running buffer.

8.3.2.2 Staining method

The RNAs were deglyoxalised before EtBr staining.

1. The gel was rinsed after electrophoresis with DEPC H_2O.
2. It was soaked in 50 mM NaOH for 15 minutes.

3. The gel was rinsed with DEPC H_2O and transferred to 0.2 M NaOAc, pH 4.8 to incubate for 15 minutes at room temperature.
4. The gel was stained with 1 μg/ml EtBr for 30 minutes.

8.3.3 Northern blotting and hybridisation

8.3.3.1 Electrophoresis

The RNA samples were separated on the glyoxal containing denaturing agarose gel. Glyoxylated RNA should be transferred immediately after electrophoresis from agarose gels to nylon membranes by capillary elution.

8.3.3.2 Northern blotting

1. The unstained gel was placed in an RNase-free dish and rinsed with deionised H_2O.
2. The gel was transferred to the platform for capillary blotting. The tank was filled with 20 × SSC for blotting the RNA onto a membrane at room temperature overnight.
3. Membrane and gel were recovered.
4. The membrane was rinsed in 2 × SSC, then air dried.
5. To immobilise the RNA on the filter, the membrane was exposed to a UV transilluminator (254-nm wavelength) for an appropriate length of time.
6. The blotted gel was stained with EtBr for 30 minutes to examine the transfer efficiency.

8.3.3.3 Hybridisation

1. A DNA or RNA probe which was labelled by DIG-dUTP or radioactivity was prepared.
2. The membrane carrying the immobilised RNA was wetted in 5 × SSC.

3. The membrane was placed (RNA-side-up) in a hybridisation tube or bag. 1 ml prehybridisation solution (Appendix) per 10 cm^2 of membrane was added.
4. The tube was incubated in the hybridisation oven at 42°C for 1 hour and rotated.
5. Double-stranded probes had to be denatured by boiling for 5 minutes.
6. The prehybridisation solution was discarded. The denatured probe was mixed with hybridisation solution.
7. The membrane was hybridised in hybridisation solution at 42°C or at a desired temperature overnight.
8. The membrane was washed by following the same procedures in Chapter 8, §1.7.3. If the probe was radioactively labelled, it was subjected to autoradiography. If it was DIG labelled, the procedures described in Chapter 8, §1.12 were followed.

8.3.3.4 Membrane staining method

Methylene blue in an aqueous solution is used for quantitative or qualitative examination of RNA and DNA immobilised on hybridisation membranes in Northern/Southern blotting. Methylene blue staining does not interfere with retention of RNA or DNA on the hybridisation membrane nor with the hybridisation process.

1. RNA or DNA was immobilised on a hybridisation membrane.
2. The membrane was immersed in methylene blue solution for 5-10 minutes at room temperature.
3. Methylene blue solution could be decanted for re-use. The membrane was washed three times with H_2O for 5-10 seconds, during which is was shaken gently.
4. The membrane was washed with 0.1-1% SDS to remove the stain.

8.3.3.5 Stripping of the probes from membranes

This protocol described the removal of the bound probe from the Northern blot membrane after chemiluminescence detection [Manual A].

1. The membrane was rinsed thoroughly in H_2O.
2. The membrane was incubated 2 × 30 minutes in 50% dimethylformamide; 1% SDS; 50 mM Tris·HCl, pH 8.0 at 68°C.
3. The membrane was rinsed first in H_2O then in 2 × SSC.
4. The stripped membrane can be stored in 2 × SSC in a sealed plastic bag if hybridisation was not carried out immediately.

8.4 Photographic techniques

8.4.1 Photographing gels on a UV light box

This technique is used to photograph DNA and RNA bands on EtBr stained agarose or acrylamide gels.

1. Panatomic F film (Ilford ; ASA 32) was loaded.
2. The gel was placed on a UV light box. The edge of the gel was used for focusing under light. A red filter was placed in front of the lens.
3. The aperture was set to the largest one available (2.8 or 4) on the camera. The exposure indicator was set on B.
4. The UV light was switched on. Different exposure times (10, 20, 30, 60, and 90 seconds) were used to take the photos.

8.4.2 Photographing gels on a light box

This protocol was followed to photograph a colour stained gel or membrane, or an X-ray film.

1. Technical Pan (Kodak) black-and white negative film was used.
2. The gel was placed on a light box for focusing. The aperture was set to the largest one and a red filter was applied to increase the contrast.

3. The light box was switched on. The exposure time was determined from the integrated light metre of the camera.
4. Different exposure times were used to ensure good contrast.

8.4.3 Developing X-ray films

Work in a dark room with red safety light.

1. X-ray film was soaked in developing solution until dark bands or other patterns became visible on the film.
2. The film was washed in H_2O or dilute acetic acid solution for 1 minute.
3. The film was then transferred to a container with fixing solution, and immersed completely for 1 minute.
4. The film was rinsed in running H_2O for 10 minutes and allowed to dry afterwards.

Appendix

Buffers and reagents

DNA techniques

Alkaline lysis buffers (Chapter 8.1.1)

P1 buffer

EDTA	10 mM
Tris·HCl, pH 8.0	50 mM
(glucose	50 mM)

P2 buffer

NaOH	0.2 M
SDS	1%

P3 buffer

KAc, pH 4.8	2.55 M

10 × CIP dephosphorylation buffer (Chapter 8.1.4)

$ZnCl_2$	100 mM
$MgCl_2$	10 mM
Tris·HCl, pH 8.3	100 mM

DIG detection system buffers (Chapter 8.1.12)

Buffer I

NaCl	150 mM
Maleic acid, pH 7.5	100 mM

Buffer II

1% blocking reagent dilute in buffer I

Blocking reagent is dissolved in buffer I to a final concentration of 10% (w/v).

Buffer III

Tris·HCl, pH 9.5	100 mM
NaCl	100 mM
($MgCl_2$	50 mM)

Do not autoclave this solution.

DIG tailing labelling 5 × reaction buffer (Chapter 8.1.5)

Potassium cacodylate	1 M
Tris·HCl, pH 6.6	0.125 M
BSA	1.25 mg/ml

Store at -20°C.

Electrophoresis solution (Chapter 8.1.3)

Acrylamide/bisacrylamide stock

A. denaturing gel (40%)

acrylamide	38 g
bisacrylamide	2 g

H_2O to 100 ml.

B. non-denaturing gel (30%)

acrylamide	29 g
bisacrylamide	1 g

H_2O to 100 ml

Elution buffer (extract DNA from acrylamide gel; Chapter 8.1.8)

Ammonium acetate	0.5 M
EDTA	1 mM

pH 8.0

Gel loading buffer (Chapter 8.1.3)

1 × TBE buffer

Bromophenol blue	0.2%
Xylene cyanol FF	0.2%
Glycerol	50%

Hybridisation solution/Prehybridisation solution (Chapter 8.1.7)

6 × SSC

5 × Denhardt's solution

0.5% SDS

100 × Denhardt's solution

BSA	2%
Ficoll	2%
PVP (polyvinyl pyrollidone)	2%

Store at -20°C.

Ligation 10 × buffer (for T4 ligase; Chapter 8.1.4)

Tris·HCl, pH 7.8	300 mM
$MgCl_2$	100 mM
DTT	100 mM
ATP	10 mM

Keep frozen.

Luria broth (Chapter 8.1.1) per litre

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
(Agar	3%)

Nuclease P1 10 × digestion buffer (buffer H; Chapter 8.1.2)

Tris·HCl, pH 7.5 (37°C)	50 mM
$MgCl_2$	10 mM
NaCl	100 mM
DTE (Dithioerythritol)	1 mM

Keep frozen.

PCR 10 × buffer (for DynaZyme; Chapter 8.1.6)

Tris·HCl, pH 8.8 (25°C)	100 mM
$MgCl_2$	15 mM
KCl	500 mM
Triton X-100	1%

Keep frozen.

Phenol (equilibrated; Chapter 8.1.1)

Before used, phenol must be equilibrated to a pH > 7.8 because DNA will partition into the organic phase at acid pH.

1. Melt the phenol at 68°C. Add hydroxyquinoline to a final conc. of 0.1%.
2. To the melted phenol, add 1 vol. of buffer (0.5 M Tris·HCl, pH 8.0 at r.t.) to the phenol. Stir the mixture on a magnetic stirrer for 15 minutes, and then turn off the stirrer. When the two phases have separated, aspirate as much as possible of the upper phase.
3. Add 1 vol. of Tris·HCl, pH 8.0 to the phenol. Repeat the same procedure in step 2. Repeat the extraction until the pH of the phenolic phase is > 7.8.
4. After the phenol is equilibrated and the final aqueous phase has been removed, add 0.1 vol. of 0.1 M Tris·HCl, pH 8.0. Store at 4°C.

10 × T4 Polynucleotide kinase buffer (Chapter 8.1.5)

Tris·HCl, pH 7.5	50 mM
$MgCl_2$	100 mM
DTT	50 mM
Spermidine	1 mM

Store at -20°C.

20 × SSC buffer (Chapter 8.1.7)

NaCl	3 M
Sodium Citrate·$2H_2O$	0.3 M

pH 7.0

10 × STE buffer (Chapter 8.1.5)

Tris·HCl, pH 7.5	100 mM
NaCl	100 mM
EDTA	1 mM

Supaquick DNA extraction buffer (Chapter 8.1.1)

Tris·HCl, pH 7.5	200 mM
NaCl	250 mM
EDTA	25 mM
SDS	0.5%

50 × TAE buffer (Chapter 8.1.3)

Tris·acetate	2 M
EDTA	0.1 M
pH 8.5	

10 × TBE buffer (Chapter 8.1.3)

Tris base	0.89 M
Boric acid	0.89 M
EDTA, pH 8.0	10 mM

10 × TE buffer (Chapter 8.1.1)

Tris·HCl, pH 7.5	100 mM
EDTA, pH 8.0	1 mM

TSB buffer (Chapter 8.1.4)

DMSO	5%
PEG (6,000)	10%
$MgSO_4 \cdot 7H_2O$	10 mM
$MgCl_2$	10 mM
(glucose	20 mM)

pH 6.1, cold sterilisation.

Protein techniques:

Column buffer (Chapter 8.2.1)

Tris·HCl, pH 7.4	20 mM
NaCl	200 mM
EDTA	1 mM

Denaturing solution (Chapter 4)

Tris·HCl, pH 8.0	10 mM
Guanidine hydrochloride	6 M

Electrophoresis (Chapter 8.2.3)

A. Acrylamide/bisacrylamide stock solution

Acrylamide	30.0 g
Bisacrylamide	0.8 g

Add H_2O to 100 ml.

5 × Electrophoresis buffer

Tris base	0.125 M
Glycine	1 M
SDS	5%

2 × SDS/sample buffer

Tris·HCl, pH 6.8	1.25 M
Glycerol	20%
SDS (w/v)	2%
Bromophenol blue	0.001%

4 × Tris/SDS buffer (pH 8.8, separating gel)

Tris·HCl, pH 8.8	1.5 M
SDS (w/v)	0.4%

Filter the solution through a 0.45-µm filter and store at 4°C.

4 × Tris/SDS buffer (pH 6.8, stacking gel)

Tris·HCl, pH 6.8	0.5 M
SDS (w/v)	0.4%

Filter solution through a 0.45-µm filter and store at 4°C.

Gel staining and destaining (Chapter 8.2.3)

Coomassie blue staining solution

Coomassie brilliant blue (w/v)	0.1%
HPLC grade methanol	50%
Glacial acetic acid	10%

Filter the solution through a Whatman no. 1 paper.

Destaining solution

Glacial acetic acid	7%
HPLC grade methanol	70%

Storing solution

Glacial acetic acid	7%

Western blotting (Chapter 8.2.4)

Blocking solution:1% PVP

Add PVP (polyvinyl pyrollidone), 40,000 to a final concentration of 1% to 1 × PBS containing 0.05% Tween 20.

10 × PBS buffer

NaCl	137 mM
KCl	2.7 mM
Na_2HPO_4	5.4 mM
KH_2PO_4	1.7 mM

Adjust the pH to 7.4 with 1 M HCl.

Transfer buffer

Tris·HCl, pH 8.3 - 8.4	25 mM
Glycine	200 mM
Methanol	15%

RNA techniques:

DEPC H_2O (Chapter 8.3.1)

DEPC (diethylpyrocarbonate)	0.2%

Shake vigorously and then autoclave.

Extraction buffer (hot phenol; Chapter 8.3.1)

Phenol	50%
Tris·HCl, pH 8.0	0.5 M
EDTA	10 mM
SDS	1%
LiCl	0.1 M

Incubate at 80°C.

Hybridisation buffer/prehybridisation buffer (Chapter 8.3.3)

DIG easy Hyb solution (Boehringer Mannheim) was used for Northern hybridisation. It is non-toxic and DNase and RNase free.

( ): optional

M: mol/litre

mM: millimol/litre

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